The Role of Chromatin in the Transcriptional Regulation of the Human T-cell Leukemia Virus Type I

by

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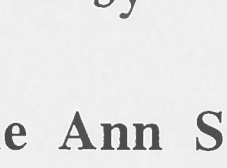
A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

of the



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STATEMENT

The research described in this thesis was solely and entirely conducted by the author unless acknowledgment is made in the text. It has not been presented for any other degree.

Nicole Siddon
September 2000

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ABSTRACT

A typical eukaryotic cell contains approximately 2 metres of DNA compacted into a nucleus of 20 μm in diameter. This packaging of DNA is achieved by a hierarchical scheme of folding and compaction into a protein-DNA ensemble called chromatin. At the first level of organisation, approximately two superhelical turns of DNA are wrapped around a protein complex consisting of eight histone molecules. This complete unit, the nucleosome, forms the basic building block of chromatin. *In vivo*, inducible gene transcription from a promoter occurs within such a chromatin context. The compaction of a gene into chromatin impedes this process of transcription. Therefore mechanisms exist in the cell to overcome the problem of chromatin compaction. Moreover, the cell utilises the structure of chromatin to specifically regulate the transcriptional activity of a gene.

In this study we employed a biochemical approach to investigate the regulation of transcription in chromatin. We used the human T-cell leukemia virus (HTLV-I) as a model system to investigate the effect of chromatin on transcription with also the aim of understanding viral gene expression at a molecular level. HTLV-I is a retrovirus which infects T cells and is the causative agent of diseases including Adult T-cell Leukemia. Of particular importance to the disease state is a viral genome encoded 40 kDa protein termed Tax. The Tax protein has been shown to interact with cellular genes that control growth and stimulation of T cells. This interaction of Tax has been proposed to cause the transformation of the host cell. In addition to its role as an oncoprotein, Tax transactivates the HTLV-I genome resulting in transcription and replication of the virus. However, Tax is not a conventional transcription factor that binds directly to DNA rather, Tax modulates transcription by interactions with existing cellular transcription factors.

The most significant of these factors in terms of Tax mediated transcription is the cyclic AMP responsive element binding protein (CREB). CREB binds to the promoters of many cellular genes where it is phosphorylated by kinases in response to extra-cellular signalling pathways to activate gene expression. Interestingly, on the viral promoter, interaction of Tax with the non-phosphorylated form of CREB is also thought to result in the transcription of the virus. The mechanism of activation employed by CREB involves interactions with the co-activator CREB Binding Protein (CBP). On the HTLV-I LTR, Tax acts as a bridge linking CREB with CBP. At the time of the start of this study, CBP was

thought to activate transcription by interaction with the basal transcriptional machinery of the cell. Early in this study however, CBP was identified as having inherent histone acetylation (HAT) activity. We therefore postulated that recruitment of CBP to the promoter targets the HAT activity of CBP directly to nucleosomes at the promoter. Acetylation of the histones may then result in transcriptional activation by the destabilisation of nucleosome structure. The interactions between Tax, CREB, CBP with the HTLV-I promoter however, have not been examined within a chromatin context.

In this study, we reconstituted DNA *in vitro* into nucleosomes using chromatin assembly systems. Inducible transcription of HTLV-I by CREB, Tax and CBP in a chromatin context was investigated. An important first step in the process of inducible transcription is the initial binding of a transcriptional activator to a nucleosomal template. Therefore, to investigate the binding of CREB to nucleosomal DNA, the first system involved assembling a 180 bp element of the HTLV-I promoter into a single nucleosome. We demonstrated that CREB could bind to its recognition site when this site was assembled into a single acetylated or non-acetylated nucleosome. The affinity of CREB binding was relatively high in comparison to other transcription factors as CREB bound to a nucleosome with only an eight-fold reduction in affinity relative to naked DNA.

To analyse the functional significance of such binding, an *in vitro* chromatin assembly and transcription system was developed. This system employed the nucleosome assembly protein-1 (NAP-1) to assemble purified nucleosomes in an array on the natural HTLV-I promoter sequence. Basal transcription from HTLV-I sequences assembled with the NAP-1 system was repressed. Purified full length proteins Tax, CREB and CBP were added to the assembly reactions to determine if these factors were sufficient for de-repression of HTLV-I. Importantly, by assessing the interaction of these proteins with naked DNA, we demonstrated that these proteins were active.

On templates assembled with a high nucleosome density, repression of basal transcription was greater then 100-fold. The recombinant factors Tax, CREB and CBP were not able to overcome the repressive effect of high density chromatin assembly. However, on less densely assembled templates (10-fold repression of basal transcription), the combination of Tax, CREB and CBP modestly (6- to 8-fold) activated transcription *in vitro*. This study is the first to demonstrate activation of HTLV-I transcription by Tax, CREB and CBP on chromatin assembled templates.

Although Tax, CREB and CBP are not sufficient for high level expression of HTLV-I in a fully chromatin assembled context, these factors can de-repress transcription from less densely assembled nucleosomal templates. This finding implies that interactions between Tax and other components of the cellular co-activator network are required for high level Tax-mediated LTR transactivation within a maximally assembled chromatin environment. Such cellular components may include chromatin remodellers that destabilise and decompact chromatin thereby allowing Tax, CREB and CBP to function. Importantly, this study opens the way for the identification of additional components required for HTLV-I transcription.

Importantly, this study reports that the activation of transcription by CBP demonstrated on less densely assembled templates is not dependent on histone acetylation as addition of CBP (in combination with Tax and CREB) modestly activated expression from the HTLV-I LTR in the absence of acetyl-coA, a factor required for acetylation activity. This result challenges the generally accepted mechanism of CBP mediated transcriptional regulation because this work suggests that the acetylation activity of CBP may not be necessary for transcriptional activation on all promoters. The use of natural promoter sequences, full length proteins and a purified chromatin assembly system represents a comprehensive first approach to the study of HTLV-I transcription within a chromatin environment.

PUBLICATIONS

Siddon, N. and Tremethick, D. J. The role of histone acetylation in regulating gene expression. *Australian Biochemist* **30**: 5-7

Johnson-Saliba, M., N.A. Siddon, M.J. Clarkson, D.J. Tremethick, and D.A. Jans. 2000. Distinct importin recognition properties of histones and chromatin assembly factors. *FEBS Lett* **467**: 169-174.

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LIST OF ABBREVIATIONS

β-ME	β-mercaptoethanol
μCi	microCurie
μg	microgram
μl	microlitre
ACF	accessibility factor
ACTR	activator for nuclear hormone receptors
Ada	adaptor protiens
AP-1	activator protien-1
ATF	activating transcription factor
ATL	adult T-cell leukemia (lymphoma)
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
bp	base pairs
BSA	bovine serum albumin
bZIP	basic DNA-binding leucine zipper
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
cDNA	complementary DNA
CHRAC	chromatin assessibility complex
CoA	coenzyme A
cpm	counts per minute
CRE	cAMP responsive element
CREB	cAMP responsive element binding protien
CTP	cytosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	elthylenediaminetetraacetic acid
EGTA	ethyl glycol-*bis*(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay

ER	estrogen receptor
ESA1	essential SAS2-related acetyltransferase
Exo III	Exonuclease III
g	relative centrifugal force
GM-CSF	Granulocyte macrophage-colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTF	general transcription factor
GTP	Guanosine triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIV-1	human immunodeficiency virus type 1
HMG	high mobility group
HSE	heat shock element
HSF	heat shock factor
HTLV-I	Human T-cell leukemia (lymphotrophic) virus
Ig	immunoglobin
IL	interleukin
INFβ	interferon β
kb	kilo base
kDa	kilo dalton
LB	Luria broth
LEF	lymphoid enhancer-binding factor
LTR	long terminal repeat
M	molar
mg	milligram
MHC	major histocompatability complex
ml	millilitre
mM	millimolar
MMTV	mouse mammary tumor virus
mn	micrococcal nuclease
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NAP-1	nucleosome assembly protein-1

NF-κB	nuclear factor-kappa B
NF-AT	nuclear factor of activated T-cells
ng	nanogram
nm	nanometre
NP-40	Nonident P-40
NURD	nucleosome remodeller and deacetylase
NURF	nucleosome remodelling factor
OD	optical density
p/CAF	p300/CBP associated factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pBS	Bluescript plasmid
PCR	polymerase chain reaction
pfu	Plaque forming units
pg	picogram
PI units	Phosphoimager units
Pit-1	
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethanesulfonyl fluoride
PNK	polynucleotide kinase
r.p.m	revolution per minute
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
rRNA	ribosomal RNA
RSC	remoddells the structure of chromatin
rt	room temperature
SAGA	Spt-Ada-Gcn5-acetyltransferase
SDS	sodium dodecyl sulphate
SPT	supressor of Ty
SRC-1	steroid receptor coactivator-1
SRE	serum response element
SWI/SNF	mating type switch/sucrose non-fermenting (co-factors)
TAF	TATA-box binding protein associated factor

TBE (1X)	90 mM Tris-borate/2 mM EDTA
TBP	TATA-box binding protein
TCR	T-cell receptor
TE	10 mM Tris-hydrochloride, 1 mM EDTA. pH 8.0
TF	transcription factor
TRE	Tax responsive element
Tris	tris(hydroxymethyl)amino-methane
tRNA	transfer RNA
TSX	tricohstatin
TTP	thymidine triphosphate
U	units

CHAPTER 1:

General Introduction

1.1 Introduction

The regulation of gene expression is of paramount importance to all aspects of life. Development of a single cell into a complex multicellular organism requires the precise temporal and spatial regulation of gene transcription. In the nucleus, genes function in a dynamic complex of DNA and protein termed chromatin. Historically, and until relatively recently, chromatin was viewed as a purely structural matrix apparently invisible to the transcriptional apparatus. Indeed, one remarkable function of chromatin is to condense and package approximately two meters of DNA into a nucleus of 20 μm in diameter. However, it is now clearly established that chromatin has a direct role in the regulation of gene expression.

As a result of the compaction of the eukaryotic genome, the conformation and accessibility of DNA is dramatically altered. It has been well documented that this chromatin compaction exherts an inhibitory effect on basal, inducible and tissue specific gene transcription. Therefore, the cell has devised mechanisms that reversibly de-compact or unfold chromatin to allow protein factor binding and transcription. Thus, compaction and de-compaction of DNA into chromatin is one of the major mechanisms by which the cell can precisely regulate gene expression.

Various approaches have been used by many investigators to gain an understanding of the mechanisms involved in the chromatin mediated control of gene expression. Such approaches include genetic, *in vitro* and *in vivo* structural analysis [most recently the Chromatin Immuno-Precipitiation (ChIP) assay] and biochemical approaches. In this research study we used a biochemical approach to understand the molecular mechanisms that underlie transcriptional control within a chromatin environment. This biochemical approach uses the Human T-Cell Leukemia Virus type I (HTLV-I) promoter as a model to examine the effect of chromatin on gene regulation, with the aim of also understanding retroviral gene expression. To understand the role of chromatin at the molecular level, a key approach in this work involves the assembly of natural viral and cellular promoters into chromatin *in vitro*. A variety of transcription factors are then added to assess their effect on binding and transcription in a defined environment. In this context, a literature review

comprising of two sections will be presented. Firstly, the issue of chromatin compaction will be reviewed including the mechanisms by which the cell uses chromatin as a means to regulate transcription. The second section looks specifically at current mechanisms thought to be involved in the control of HTLV-I transcription.

1.2 Chromatin Structure

Within the nucleus, chromatin is made up of DNA and protein in roughly an equal mass ratio. The protein component of chromatin is divided into two classes, histone proteins and non-histone proteins.

1.2.1 Histone proteins

The histone proteins form the protein scaffold onto which the DNA is wrapped to produce the nucleosome. It consists of the nucleosome core (147 bp of DNA wrapped around a histone octamer), the linker histone H1 and the linker DNA between the nucleosome cores (Kornberg, 1974). The nucleosome is the primary building block of chromatin. Numerous details of nucleosome structure have been revealed over the past 25 years. These discoveries culminated in solving the high-resolution crystal structure of the nucleosome core (Luger *et al.*, 1997) (figure 1.1).

(a) Structure of the nucleosome core

The nucleosome core is made up of an octamer of the four core histones in a tripartite assembly in which a centrally located tetramer of (H3-H4) is flanked by two H2A-H2B dimers (Kornberg and Thomas, 1974; Camerini-Otero *et al.*, 1976; Eickbush and Moudrianakis, 1978) (figure 1.1). This tripartite structure is physiologically significant as the process of chromatin assembly *in vivo* occurs by initial deposition of a H3-H4 tetramer followed by the incorporation of two H2A-H2B dimers (Worcel *et al.*, 1978; Jackson, 1987). Each histone consists of a structured globular domain called the histone fold, as well as two unstructured tails (figure 1.2).

DNA (146 bp), is wrapped around the histone octamer in 1.65 turns of a flat, left handed superhelix (Richmond *et al.*, 1984) (figure 1.3). Compared to DNA in solution, wrapping of the DNA around the nucleosome core changes properties of the DNA, which has important implications for the control of gene expression. For example, the path of the

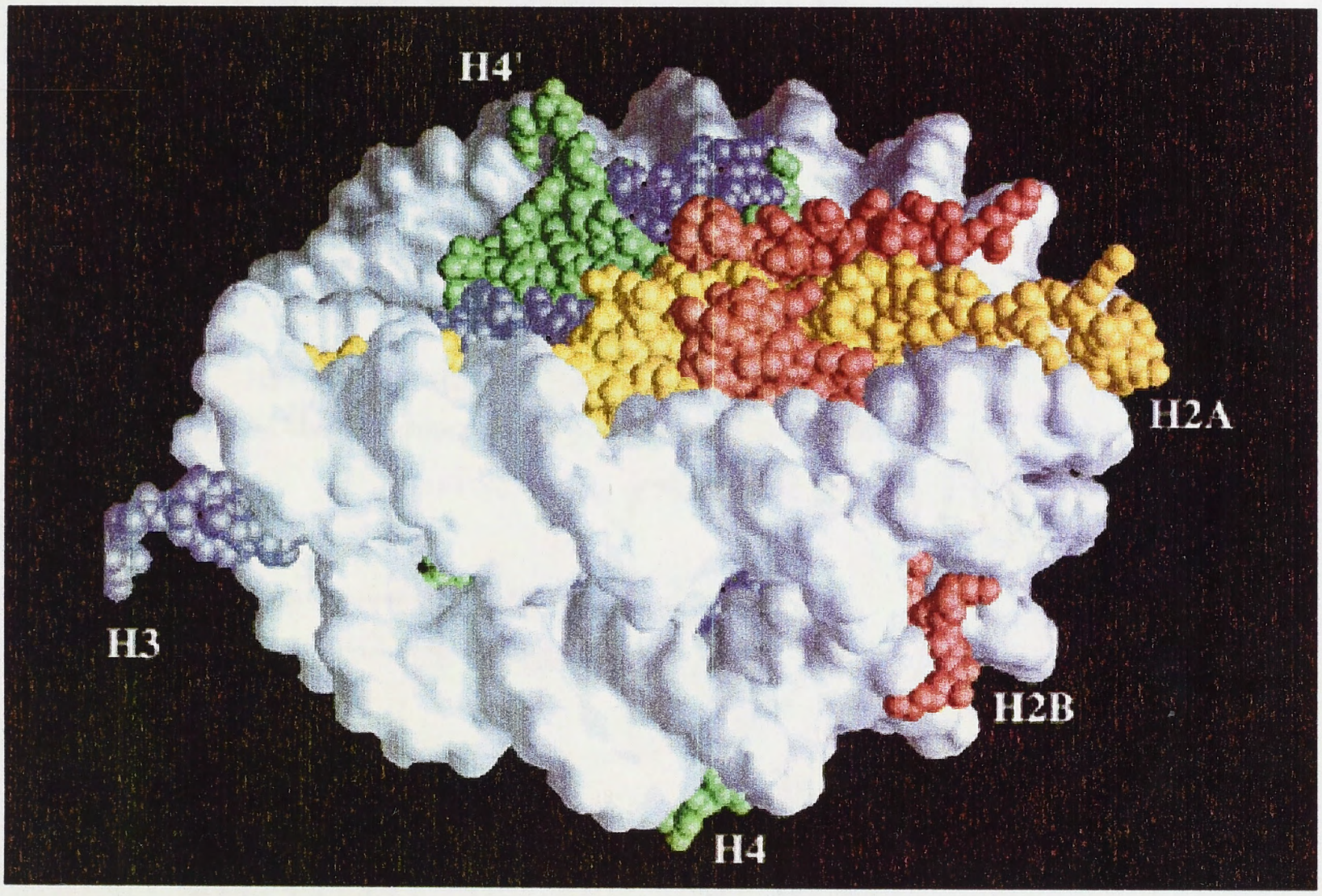


Figure 1.1: Crystal structure of the nucleosome core particle. The X-ray crystal structure of the nucleosome core particle of chromatin shows in atomic detail how the histone protein octamer is assembled. 146 bp of DNA (white) is organised in a superhelix around the histone octamer consisting of four histone 'fold dimers' defined by H3-H4 and H2A-H2B histone pairs. Histone H4 is green, histone H3 in blue, histone H2A in yellow and H2B in red. Figure sourced from Luger *et al.* (1997), (http://www.mdanderson.org/~genedev/Bone/nucstruct.html).

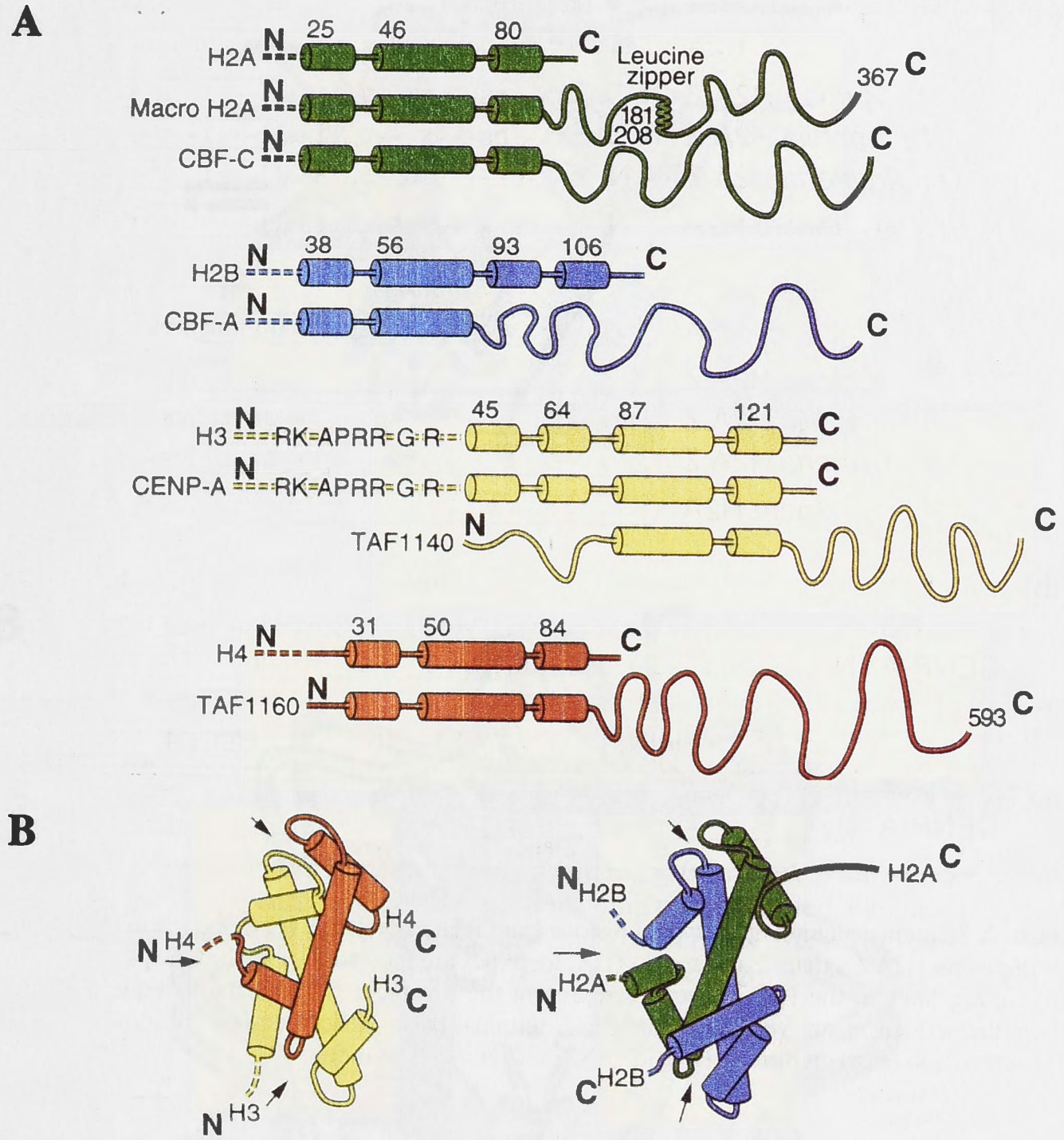
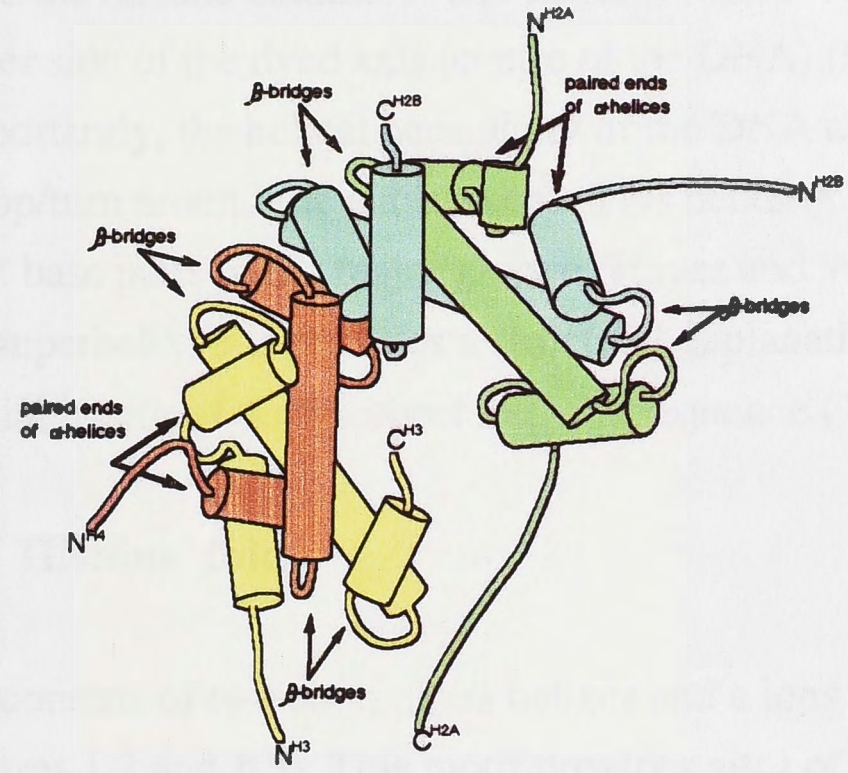


Figure 1.2: The histone fold of histones and regulatory proteins and the handshake motif. A. The core histones are shown in a linear representation with the approximate regions of the α-helix shown as cylinders. Numbers indicate the first amino acid relative to the amino terminus of the histone protein for each helix. The related proteins are shown below each histone. Proteins related to histone H2A are in green, to histone H2B in blue, to histone H3 in yellow and to histone H4 in red. The entire N-terminal tail of the core histones is not drawn to scale. **B**. The heterodimerisation domains of histones H3 and H4 and of histones H2A and H2B are shown coloured as in panel A. The interaction between the histone fold domains is described as a handshake. The sites of interaction with DNA within the heterodimer are indicated by the arrows. The positions of the N-terminal tails of the core histones are shown as dashed lines. Figure sourced from Wolffe and Pruss, (1996).

A



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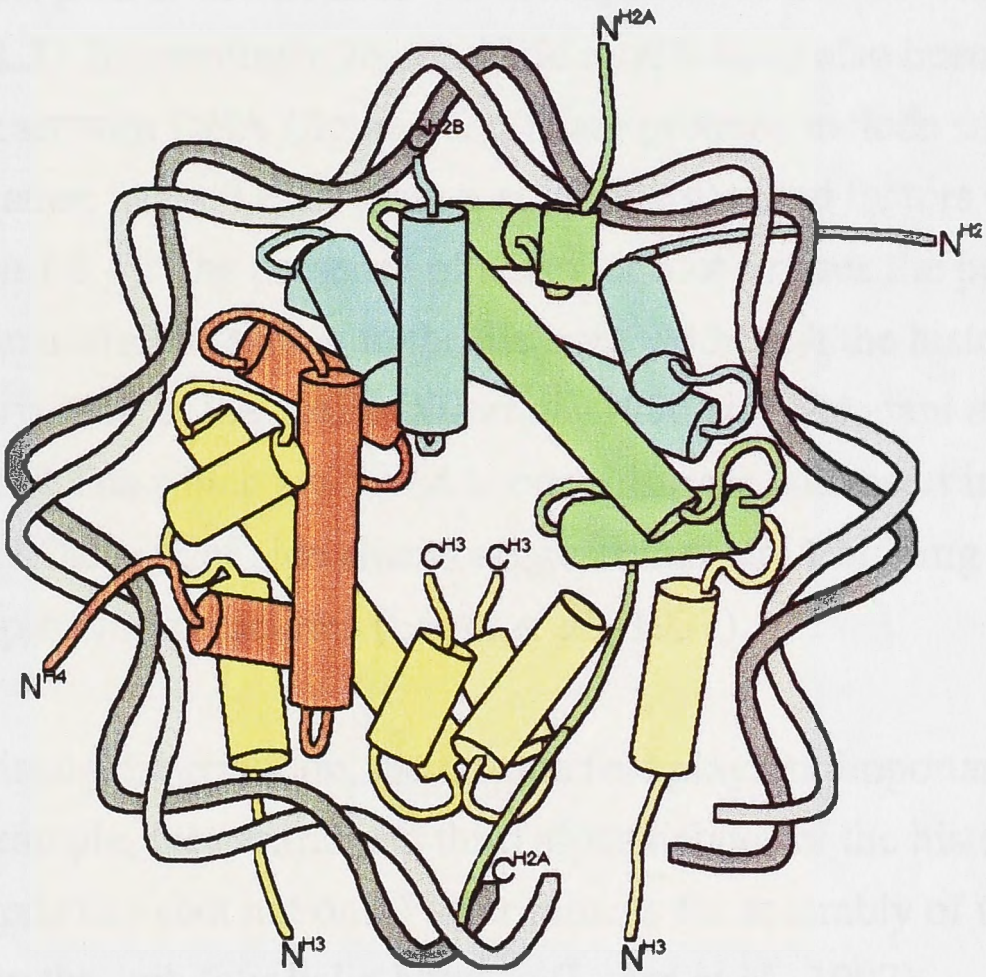


Figure 1.3: organisation of the histone core. A. The heterodimerisation domains of the core histones shown in figure 1.2 form the building block for the assembly of the histone core. Histone H2A is in green, histone H2B in blue, histone H4 in red and the H3 histones are in yellow. **B.** 146 bp of DNA are wrapped around the histone core in 1.65 turns of a left handed superhelix. The DNA contacts the histone core every 10 bp. Core histones are coloured as for panel A. Figure sourced from D. Pruss (http://www.average.org/~pruss/nucleosome.html).

superhelix around the histone octamer is not uniform with severe bending at about 10 bp and 40 bp to either side of the dyad axis (centre of the DNA) (Luger *et al.*, 1997; Richmond *et al.*, 1984). Importantly, the helical periodicity of the DNA changes from 10.5 bp/turn in solution to 10.2 bp/turn around the nucleosome. This bending alters the separation of the edges of adjacent base pairs in the major groove (Hayes and Wolffe, 1992). Together with distortion in the superhelix, this provides a structural explanation for why nucleosomes take up preferential positions with respect to DNA sequence (Travers and Klug, 1987).

(b) The Histone fold

The histone fold consists of two short alpha helixes and a long central helix separated by beta bridges (figures 1.2 and 1.3). This motif provides sites of histone DNA interaction as well as providing for the dimerization of histones via a handshake motif, in which each monomer clasps its partner in a head-to-tail arrangement (Arents and Moudrianakis, 1995) (figures 1.2 and 1.3). Interestingly, histone fold motifs have also been located in other proteins that interact with DNA (figure 1.2). These proteins include subunits of the general transcription initiation factor TFIID known as TBP associated factors (TAFs) (Baxevanis *et al.*, 1995) (section 1.3.1). The presence of a similar motif raises the possibility that TAFs may wrap DNA in a similar fashion to the histones. Although the histone fold appears to be involved in dimerization of the TAFs (*Xie et al.*, 1996 and Nakatani *et al.*, 1996), TAFs lack arginine side chains which are found in core histones and insert into the minor groove of the DNA helix. The lack of side chains suggests that DNA binding by TAFs is by different protein-protein interactions (Luger *et al.*, 1997).

In addition to assisting dimerization, the histone fold plays an important role in octamer formation. For example, the central and third alpha helices of the histone folds of H4, H3 and H2A are the primary (but not only) determinants for assembly of the tetramer and octamer formation through four helix bundles (Luger *et al.*, 1997).

(c) Histone tails

The histone tails comprise the unstructured component of histones within the context of the individual nucleosome. However, despite this lack of structure (in the context of a single nucleosome) it has become increasingly apparent that the histone tails play a vital role in nucleosome structure and gene regulation. Interestingly, the N-termini of histones H3 and H4 are the most conserved portions of these highly evolutionarily conserved proteins

indicative of their functional importance. Because of their lack of structure, the precise arrangement of the tail in the histone octamer is difficult to determine. However, crystal structure analysis (Luger *et al.*, 1997) has provided new insights into the nature of these tails and their involvement in internucleosomal interactions. A helical periodicity of 10.2 bp, created by wrapping DNA around a nucleosome (section 1.2.1a), allows the minor grooves from neighbouring turns of the DNA to line up, forming channels through which histone tails can pass. The N-terminal tails of both H3 and H2B pass through these minor groove channels such that there is a protruding tail every 20 base pairs. Furthermore, the tails of H3 extend considerably beyond their exit points at the nucleosome DNA surface and are fixed in position by nucleosome-nucleosome contacts. Interestingly, one of the protruding tails of H4 makes many contacts with the face of the H2A-H2B dimer of a neighbouring nucleosome core.

An important aspect of the histone tails is their ability to be post-translationally modified. This modification in thought to be a crucial mechanism involved in the regulation of gene expression by chromatin structure and is discussed in section 1.4.2.2

(d) Histone H1

Histone H1 and its variants, which include H1° and H5, are referred to as linker histones because they are associated with linker DNA between the nucleosome cores. Unlike the core histones, the linker histones are less conserved in their primary structure. H1 in most organisms has three distinct domains, a central globular region, a basic N-terminal region and a C-terminal tail. Interestingly, the central globular domain exhibits a winged-helix structure which constitutes the DNA binding domain of a diverse set of proteins that interact with chromatin (Ramakrishnan *et al.*, 1993).

The precise location of H1 in chromatin is still a matter of controversy in current literature. H1 is generally thought to bind DNA at the nucleosome across the dyad axis at the linker DNA as it enters and leaves the nucleosome (Ali and Singh, 1987). However some investigators have shown evidence using the 5S rRNA gene that HI binds specifically to DNA in an asymmetric fashion (Hayes and Wolffe, 1993; Hayes, 1996; Pruss *et al.*, 1996).

A variety of structural roles have been attributed to linker histones. For example, the binding of histone H1 *in vitro*, has been shown to stabilise octamer formation by

completion and locking of the two DNA turns within the nucleosome (Garrard, 1991). In addition, H1 is thought to be involved in determining average nucleosome spacing and is proposed to facilitate the folding of nucleosomal arrays into 30 nm chromatin fibres *in vivo* (section 1.2.3) (Felsenfeld and McGhee, 1986; Kamakaka and Thomas, 1990; Tremethick and Frommer, 1992). In addition to its structural function, histone H1 has been proposed to have regulatory functions that will be discussed in section 1.4.3

1.2.2 Non-histone proteins

(a) High mobility group proteins

Although the compacted structure of DNA in eukaryotes is determined by core and linker histones, non-histone proteins make important additional contributions. Remarkably, a majority of these non-histone proteins have an unknown function, and to date, the best characterised are the High Mobility Group (HMG) proteins. HMG proteins are among the most abundant and ubiquitous non-histone proteins found in the nuclei of all higher eukaryotes. HMG proteins are divided into three distinct families, the HMG box containing HMG1/HMG2, the postulated active chromatin-associated HMG14/17 and the HMG-I(Y) proteins. HMG proteins from different sub-classes are not related to each other, and have been commonly termed HMG proteins only because of their similar extraction and solubility properties (Paranjape *et al.*, 1994). During the course of this work, interactions between HMG-I(Y) proteins and the HTLV-I LTR were detected and preliminary experiments were performed to understand the physiological role of this interaction. Thus a review of HMG-I(Y) is relevant and presented below.

There are currently three members of the HMG-I(Y) family: HMG-I, HMG-Y and HMGI-C. HMG I and Y are isoforms, which are produced by alternate splicing and are referred to collectively as HMG-I(Y). The other member of the family, HMGI-C, is expressed as a separate gene product but is structurally related (Bustin and Reeves, 1996). HMG-I(Y) proteins are characterised by three, tandemly organised, basic binding modules separated by a flexible linker. These modules are referred to as AT-hooks because in most cases they preferentially bind to the minor groove of AT rich sequences (Reeves and Nissen, 1990). Because of their binding preference to structure rather than sequence, and their ability to alter the topology of DNA, the HMG-I(Y) proteins have been termed architectural proteins.

Recently HMG-I(Y) has come to the fore as an important regulatory factor (Chin *et al.*, 1998). However it is significant to note that HMG-I(Y) does not have an intrinsic ability to regulate transcription, but rather regulates the affinity and activity of other transcription factors by altering chromatin structure. The most extensively characterised example of a region responsive to HMG-I(Y) is the β-interferon (INF-β) promoter. Meticulous experiments, over many years, conducted by Maniatis and colleagues have elucidated the role of HMG-I(Y) and other proteins in this system with great detail. In this system HMG-I(Y) enhances INF-β transcription by altering the structure of the DNA (Falvo *et al.*, 1995), allowing cooperative recruitment of the INF-β gene activators which form a stereospecific complex termed an enhanceosome (Thanos and Maniatis, 1995; Tjian and Maniatis, 1994). The formation of the enhanceosome activates transcription at high levels by prompting multiple rounds of reinitiation and transcription. (Yie *et al.*, 1999).

HMG-I(Y) function is further controlled and complicated by the observation that these proteins can be post-translationally modified by phosphorylation, methylation and acetylation (this work and Munshi *et al.*, 1998; Banks *et al.*, 2000). These modifications alter the properties of HMG-I(Y) *in vivo* and *in vitro*. In the case of the IFN-β enhanceosome, acetylation of HMG-I(Y) by CBP results in a decrease in its DNA binding affinity and its subsequent detachment from the enhanceosome (Munshi *et al.*, 1998). Recent work by Banks *et al.*, (2000) has demonstrated differential *in vivo* phosphorylation of HMG-I(Y) in two cells lines that are at different stages of neoplastic progression. Interestingly, these modifications influence the ability of HMG-I(Y) to interact with AT rich DNA substrates and nucleosome core particles *in vitro*.

Another important aspect of HMG-I(Y) proteins is that they are thought to play critical roles in cell growth and transformation. For example, HMG-I(Y) proteins are expressed at low or undetectable levels in adult tissues but are expressed highly in rapidly proliferating cells such as early embryonic cells (Chiappetta *et al.*, 1996) and neoplastic tissues (Giancotti *et al.*, 1987; Johnson *et al.*, 1988). Recently HMG-I(Y) has also been linked to viral diseases as it was shown to be a host specific factor required for the integration of HIV-1 (Farnet and Bushman, 1997; Hindmarsh *et al.*, 1999). In addition, work carried out in this laboratory has provided strong evidence for a role of HMG-I(Y) in HIV-1 transcription (Henderson *et al.*, 2000). Thus, just as the importance of histones in gene regulation has recently been appreciated, the non-histone protein HMG-I(Y) also appears to have

important regulatory roles and will be an area of considerable future research to elucidate the mechanisms involved.

1.2.3 Higher order compaction

Although the most fundamental level of chromatin organisation is the 10 nm nucleosome containing chromatin fibre, the bulk of chromatin in an interphase nucleus is present as a 30 nm diameter filament. The 30 nm filament is a 40-50 fold compaction of DNA and is a dynamic structure that folds and unfolds according to the state of transcriptional activity. The detailed structure of the 30 nm fibre remains unknown although several models have been proposed including the solenoid model, the supercoiled spacer model and the cross-linker model (reviewed in Paranjape *et al.*, 1994). A common element of these models is the importance of histone H1 in 30 nm filament formation and stabilisation. More research is urgently required to ascertain the mechanisms of, and effect of higher order compaction on gene expression. Although higher order compaction clearly plays a role in the control of gene expression, it is beyond the scope of this thesis and will not be further discussed here. However, it is worth noting that *in vitro* studies on chromatin templates, including this study, utilise $MgCl_2$ concentrations of around 5 mM. Such $MgCl_2$ concentrations effectively compact chromatin.

1.3 Transcription

The initiation of mRNA synthesis is a primary control point in the regulation of differential gene expression. Therefore in order to appreciate the importance of chromatin assembly and compaction on gene expression it is necessary to review the process of transcription.

1.3.1 General Transcription Machinery

For RNA polymerase II to transcribe a gene, an array of over 50 proteins must be assembled at the promoter. This structure has been termed the preinitiation complex (PIC). A schematic model for assembly of the PIC is shown in figure (1.4). This model is based largely on kinetic assays using purified or partly purified transcription factors, native gel electrophoresis and nuclease protection assays (Buratowski *et al.*, 1989; Conaway and Conaway, 1993; Zawel and Reinberg, 1993). The initial step is TFIID binding to the TATA element of a promoter. TFIID is a multiprotein complex consisting of the TATA-binding protein (TBP) and an array of TBP associated factors (TAFs). The TATA-TFIID

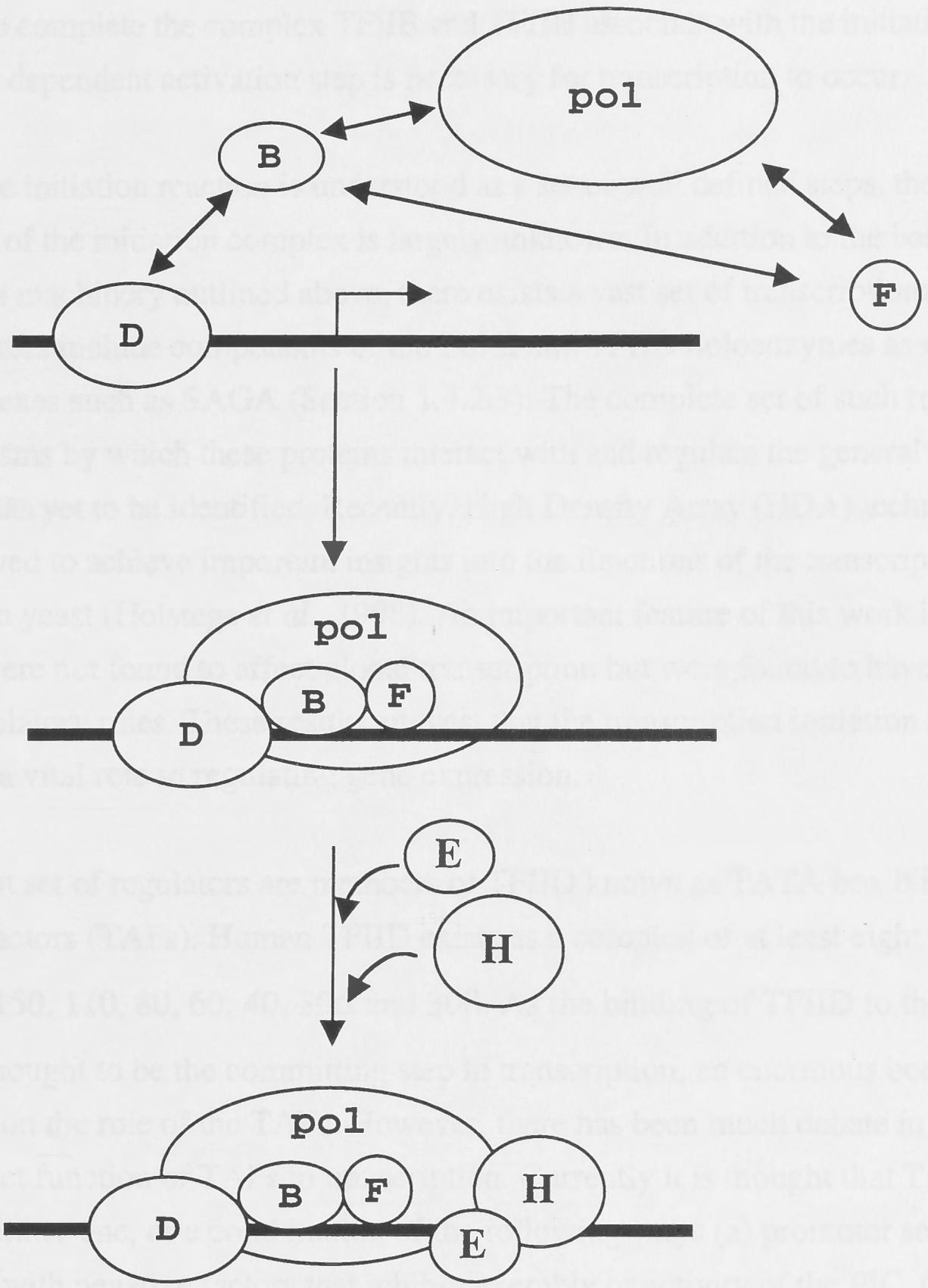


Figure 1.4: Schematic model of transcription initiation complex assembly.
Transcription factors are represented by their letter designations. The bold line represents the promoter DNA while the bent arrow indicates the initiation site. Double-headed arrows indicate protein-protein interactions. See text for details. Figure sourced from Buratowski, (1994).

complex then acts as a binding site for TFIIB, which then recruits pol II and TFIIF into the complex. To complete the complex TFIIE and TFIIH associate with the initiation complex and an ATP dependent activation step is necessary for transcription to occur.

Although the initiation reaction is understood as a set of well defined steps, the detailed architecture of the initiation complex is largely unknown. In addition to the basic transcription machinery outlined above, there exists a vast set of transcriptional regulators. Such regulators include components of the Pol II and TFIID holoenzymes as well as other large complexes such as SAGA (Section 1.4.2.3). The complete set of such regulators and the mechanisms by which these proteins interact with and regulate the general transcription machinery has yet to be identified. Recently, High Density Array (HDA) technology has been employed to achieve important insights into the functions of the transcriptional machinery in yeast (Holstege *et al.*, 1998). An important feature of this work is that certain regulators were not found to affect global transcription but were found to have gene specific regulatory roles. These results suggest that the transcription initiation apparatus itself, plays a vital role in regulating gene expression.

An important set of regulators are members of TFIID known as TATA box binding protein associated factors (TAFs). Human TFIID exists as a complex of at least eight TAFs, TAFII250, 150, 110, 80, 60, 40, 30α and 30β. As the binding of TFIID to the TATA element is thought to be the committing step in transcription, an enormous body of work has focused on the role of the TAFs. However, there has been much debate in the literature as to the exact function of TAFs in transcription. Currently it is thought that TAFs may function in either one, or a combination of the following ways (a) promoter selectivity, (b) competition with negative factors that inhibit assembly or activity of the PIC, (c) increase in the rate of transcription reinitiation by stabilising factors left behind at the promoter, (d) protein-protein interactions with other members of the general transcription machinery. (e) targets of activation domains of general transcription factors assembled on the enhancer resulting in transcriptional activation. Hahn, 1998).

1.3.2 Enhancers and transcription factors

Transcriptional activation in response to extracellular signals, or during development, involves not only the basal transcription machinery outlined above, but also the regulated assembly of multiprotein complexes on enhancers and promoters. Transcription factors, in

response to stimuli, recognise their cognate binding sites on promoters and enhancers which results in the activation of transcription. Transcription factors are composed of modular components. For example, a typical transcription factor contains a specific DNA binding domain, a multimerization domain and a transcriptional activation domain. Similarly, enhancers contain distinct sets of transcription factor binding sites. This modular structure allows for a great deal of specificity in gene regulation. Variations in the arrangement of binding sites provides the potential to create unique complexes bound to enhancers with different interacting interfaces and results in a high degree of specificity and potential for multiple regulatory controls.

Once assembled on the enhancer, the activation domains of the transcription factors are thought to interact with the basal transcriptional machinery to activate transcription directly or indirectly via co-activators. Several different types of activation domains have been identified and classed as acidic, glutamine rich, and proline rich. Remarkably, the precise molecular mechanisms of these activation domains remain obscure. There are many potential targets for the activators in the general transcription machinery. For example the activation domain of a yeast transcription factor VP16 interacts with TBP (Stringer *et al.*, 1990). However the same activation domain was also found to interact with TFIIB (Lin and Green, 1991). It is becoming increasingly apparent that a key target for activator interaction is the TAFs. For example, stimulation of *in vitro* transcription by activators can be detected with partially purified TFIID but not with purified TBP (Pugh and Tjian, 1990). In addition, several activator-TAF interactions have been documented including Sp1 interaction with TAFII110 (Hoey *et al.*, 1993; Gill *et al.*, 1994) and VP16 with TAFII40 (Goodrich *et al.*, 1993). It is important to note, however, that the function and mechanisms of action of most the TAFs have yet to be determined (section 1.3.1) and the functional relevance of the protein-protein interactions between TAFs and activators remains to be demonstrated.

It is important to keep in mind that the process of activated transcription must take place in a chromatin context. Recently it has been shown that one aspect of transcription factor function may involve interaction with, and recruitment of co-factors. These co-factors are often components large protein complexes including, SAGA, NURF, and CBP. Upon recruitment, these protein complexes are thought to remodel chromatin structure and are discussed in section 1.4.2.3)

1.4 Chromatin as a regulator of transcription

There is little doubt that chromatin, from a structural point of view, represses the expression of genes. Therefore an important question is how does transcription occur within the context of nucleosomes. Elegant genetic experiments by Grunstein and colleagues have provided pioneering evidence of the importance of nucleosomes as regulators of transcription. For example, Grunstein showed that inhibition of nucleosome formation by the repression of histone H4 levels activates several yeast promoters in the absence of inducers (Han and Grunstein, 1988; Durrin *et al.*, 1992). More recently, it was demonstrated that point mutations in specific tyrosine residues essential for the interaction between H4 and H2A-H2B dimers, can have both positive and negative effects on *in vivo* transcription (Santisteban *et al.*, 1997). Mutations in individual histones that have a specific consequence for the expression of particular genes, provides direct evidence that DNA packaging into chromatin contributes to the specific regulation of transcription.

Numerous other studies have supported a role for histones in gene regulation. For example Vetesse-Dadey *et al*, (1994) have shown that tryptic removal of the N-terminal of the core histones results in the increased *in vitro* binding of a classical transactivator, GAL4-AH to a nucleosome binding site. This result implicates the nucleosome in the control of activated transcription. This has been further supported by *in vivo* studies which demonstrate that N-termini of histones H3 and H4 are required for a1-α2 repression, a regulatory system in yeast that is involved in distinguishing diploid cells from haploid (Huang *et al.*, 1997). A positive role of nucleosomes in the regulation of gene expression has also been shown (Schild *et al.*, 1993). These investigators have demonstrated that the binding of a nucleosome on the vitellogenin B1 promoter activates transcription 8 fold relative to naked DNA. This finding implies that the correct formation of a nucleosome in a promoter region can be important for appropriate gene activation. Collectively, work by these investigators and many others, shows that rather than being merely a passive structural barrier to transcription, the nucleosome is an integral component and regulator of transcriptional mechanisms.

There appears to be two distinct strategies that nucleosomes employ to regulate gene transcription. These are firstly the persistent displacement or exclusion of nucleosomes on inducible genes. Genes of this type are known as preset genes. The second strategy

involves the direct or indirect remodelling of nucleosomes by factors that result in transcription factor access to the required cis elements.

1.4.1 Preset genes

Preset genes create an open conformation of chromatin set for the binding of factors. There are at least three mechanisms that enable genes to be set in an open conformation with the limitation that relatively few genes have been mapped *in vivo*. One way in which an open conformation can be achieved is by positioning a transactivator binding site precisely into a non-nucleosomal region of a promoter. This positioning enables the cell to side-step the nucleosomal inhibition of factor binding. A second mechanism used to generate an open conformation is the use of factors such as GAGA. A highly characterised example of this mechanism is the *Drosophila* heat shock genes, hsp26 and hsp70 (Thomas and Elgin, 1988). On the heat shock genes the GAGA factor is thought to establish or maintain the nucleosome free regions that are associated with important gene regulatory sequences (Lu *et al.*, 1993). GAGA binding elements have also been located upstream of other *Drosophila* genes including histone H3, H4, Ultrabithorax and Kruppel (Biggin and Tjian, 1988; Gilmour *et al.*, 1989; Croston *et al.*, 1991). The presence of these GAGA sites indicate that the creation of a nucleosome free environment may be a common mechanism for allowing transcription. In a third mechanism, specific DNA sequences can act to exclude nucleosomes thus keeping the promoter in an open conformation for transcriptional activation.

1.4.2 Mechanisms which alter chromatin compaction

The second way that the transcription machinery may access a chromatin template in a promoter specific way is by targeting regulatory proteins that modify chromatin structure to particular promoters. Two broad groups of enzymes have evolved that fulfil this function in the cell. One family consists of a number of different ATP-dependent multi-subunit protein complexes that can destabilise or remodel the nucleosome. The second family of enzymes that can alter the structure of chromatin are the histone acetyltransferases, or HATs, which post-translationally acetylate histones. This acetylation reaction is reversible through the action of histone deacetylases or HDAs (figure 5.1a). Both groups of enzymes may work together in a co-operative manner to achieve an open chromatin conformation. Both of these classes exist within cells as large multi-protein machines that also include many transcriptional co-factors (section 1.3.1). This finding is significant because these

machines directly link these enzymatic activities to chromatin and transcriptional regulation, highlighting the direct link between transcriptional activation and chromatin.

1.4.2.1 ATP dependent chromatin remodelling machines

Chromatin remodelling machines are a group of large multi-subunit complexes. They are related by their ability to destabilise nucleosomes by using energy in the form of adenosine tri-phosphate (ATP). The chromatin modifying machines identified to date can be categorised into three classes based on the identity of the central ATP dependent chromatin remodeller or 'engine' (Travers, 1999a). The yeast proteins swi/snf and RSC, *Drosophila* BRM and mammalian BRG1 or hBrm, all contain swi2 homologues as their engine. On the other hand, the *Drosophila* complexes NURF, CHRAC and ACF contain ISWI homologues. A third class of remodellers are CHD1/CHD3 and contain Mi2 as the putative remodelling engine. In each three classes the ATP binding sub-unit is thought to act as a processive, ATP driven DNA translocating motor that disrupts histone DNA interactions (Kornberg and Lorch, 1995; Pazin and Kadonaga, 1997). *In vitro*, all the chromatin remodelling activities containing ISWI or swi2 can increase the accessibility of nucleosomal DNA to transcription factor or other DNA binding proteins. In the case of the swi/snf complex, there is substantial genetic and biochemical evidence that complex mediated transcriptional activation is accompanied by nucleosome disruption *in vivo*. However it remains unclear whether disruption rather than restoration is the principal *in vivo* function of the majority of the complexes (Travers, 1999). Although these complexes all have the function of ATP-dependent chromatin remodelling, the other sub-units within the complex are though to be involved in their precise function and targeting within the cell (section 1.4.2.3).

1.4.2.2 Histone modifying enzymes

(a) Modification of chromatin structure by histone acetylation

X-ray crystal analysis of nucleosome structure has shown that the N-terminal tails of histones project away from the nucleosome core and therefore have the potential to interact with either regulatory proteins, linker DNA or other nucleosomes (Section 1.2.1; Luger *et al.*, 1997). These tails are characterised by a high content of the positively charged amino acids arginine and lysine, and therefore can interact strongly with DNA (Luger *et al.*,

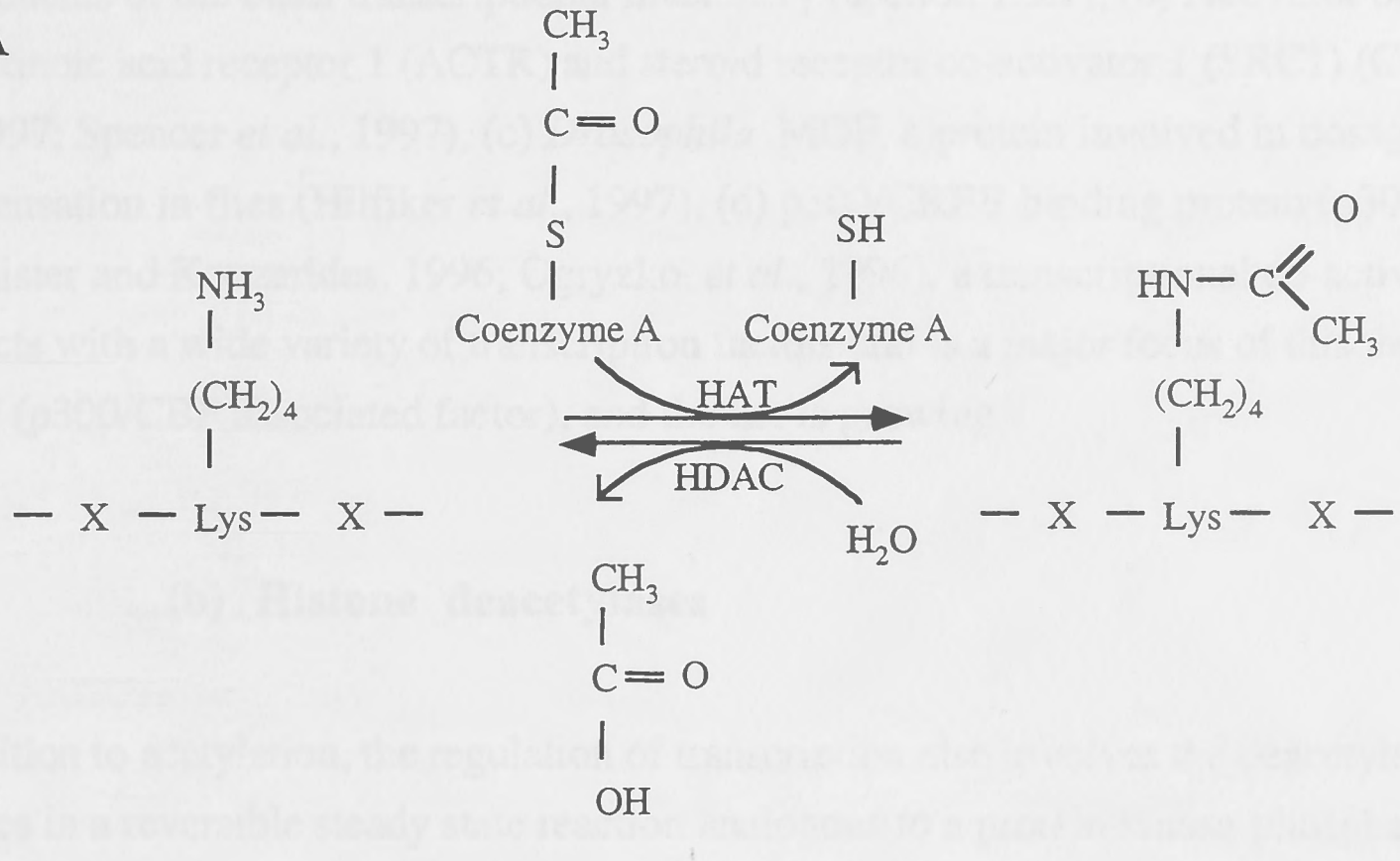
1997). Histone acetylation occurs post translationally on the ε-NH3 groups of specifically located lysines in all histones and neutralises the positive charge of the tails (figure 1.5). This charge neutralisation is thought to decrease the affinity of the tails for DNA . A critical functional role for these acetylations is indicated by a high degree of conservation of the relative positions of these lysines across eukaryotic species.

More than 30 years ago it was noted that transcriptionally active cells were enriched in acetylated histones (Allfrey *et al.*, 1964). However, until relatively recently, all reports were correlations and proof that histone acetylation was involved in the transcription process was lacking (Csordas, 1990). Stronger evidence that histone acetylation is directly involved in transcriptional activity came from the use of anti-sera specific for ε-N-acetyl lysine. Crane-Robinson and co-workers demonstrated that acetylated histones were preferentially associated with actively transcribed β-globin sequences but not with repressed ovalbumin gene sequences (Hebbes *et al.,* 1994). Similar studies have shown that in *Drosophila*, the male X chromosome is hyperacetylated, particularly on lysine 16 of H4. This hyperacetylation correlates with the high transcriptional activity of the male X for dosage compensation (Bone *et al.*, 1994). Evidence that acetylation has a causative role in transcription as been derived from *in vitro* chromatin assembly studies. For example, nucleosomal templates reconstituted with hyperacetylated histones are able to bind transcription factors more efficiently (Lee *et al.*, 1993; Ng *et al.*, 1997).

A direct demonstration that histone acetylation is intimately associated with the transcriptional process has come from the discovery that many previously described transcriptional co-factors are also histone acetyltransferases (HATs). The first nuclear HAT cloned was p55 from Tetrahymena thermophila by Brownell and Allis (Brownell *et al.*, 1996). These investigators cleverly noted that p55 is highly homologous to the yeast transcriptional activator Gcn5p which is part of a multi- subunit complex required for the expression of several genes *in vivo* (Georgakopoulous and Thireos, 1992). Subsequently, Gcn5p was shown to possess HAT activity and that this acetylation activity is critical for gene activation *in vivo* (Kuo *et al.*, 1998). These findings have definitively shown that the acetylation status of nucleosomes is a key regulatory point of transcription.

Based on the finding that Gcn5p displays HAT activity, a surprisingly large number of diverse transcription factors have been identified as possessing intrinsic HAT activity. Such proteins include (a) hTAFII130/250 and TFIIC (Mizzen *et al.*, 1996; Hsieh *et al.*, 1999),

A



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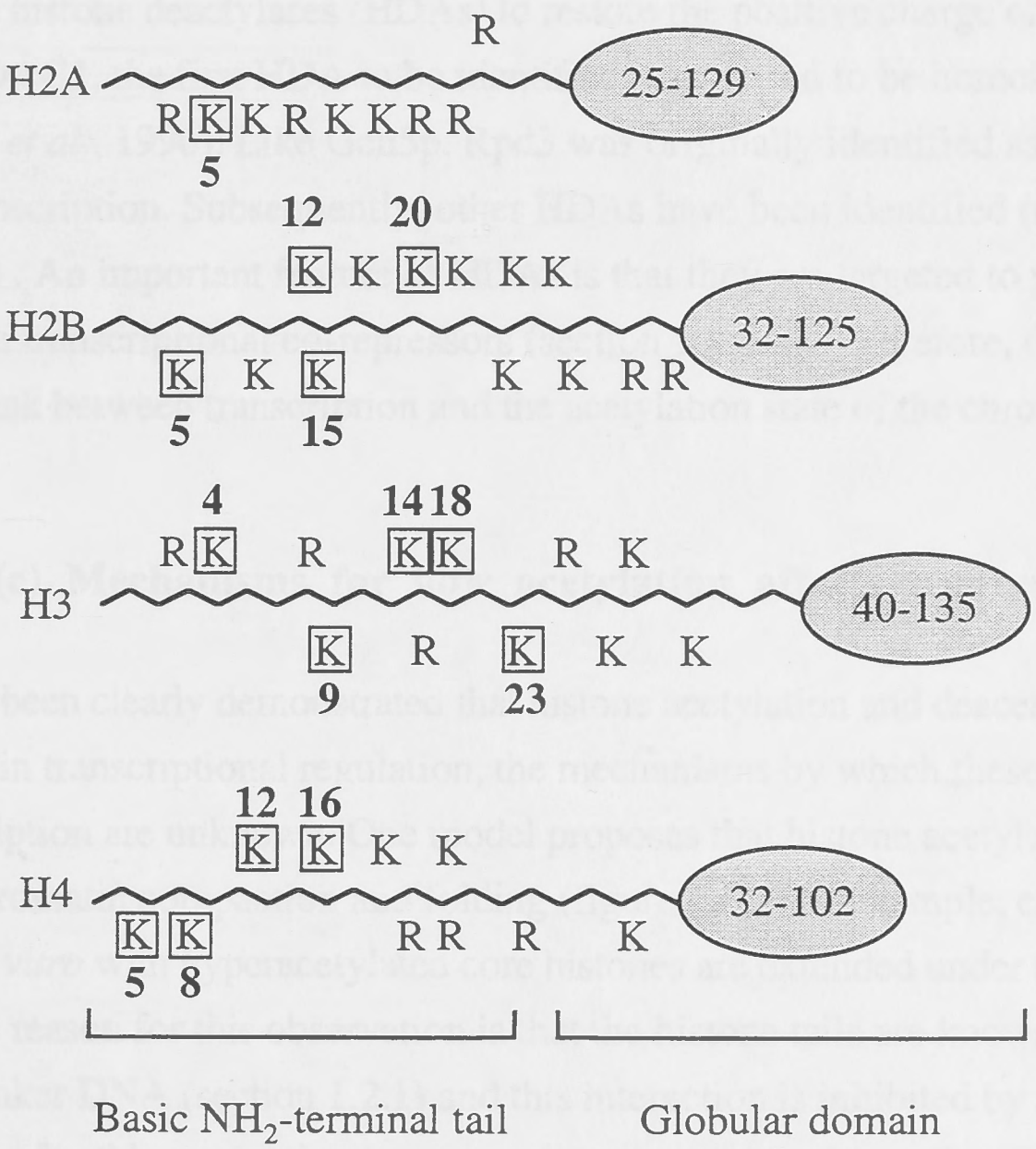


Figure 1.5: Acetylation of the core histone proteins. A. The acetylation and deacetylation reaction of a lysine amino acid (lys) on a core histone N-terminal tail. The steady state level of histone acetylation is determined by the opposing activities of histone acetyltransferases (HAT) and deacetylases (HDAC). **B.** HATs acetylate specific lysines on all four core histone tails. Sites known to be acetylated are indicated by a box and are numbered relative to the N-terminus of each core histone. The N-terminal tail is not drawn to scale.

components of the basal transcriptional machinery (section 1.3.1), (b) Activator of thyroid and retinoic acid receptor 1 (ACTR) and steroid receptor co-activator 1 (SRC1) (Chen *et al.*, 1997; Spencer *et al.*, 1997), (c) *Drosophila* MOF, a protein involved in dosage compensation in flies (Hilfiker *et al.*, 1997), (d) p300/CREB binding protein (p300/CBP) (Bannister and Kouzarides, 1996; Ogryzko, *et al.*, 1996), a transcriptional co-activator that interacts with a wide variety of transcription factors and is a major focus of this thesis, (e) pCAF (p300/CBP associated factor), and the list is growing.

(b) Histone deacetylases

In addition to acetylation, the regulation of transcription also involves the deacetylation of histones in a reversible steady state reaction analogous to a protein kinase-phosphatase reaction (figure 1.5). This reaction involves the removal of the acetate group from the ε-N-acetyl lysine by histone deactylases (HDAs) to restore the positive charge of the histone tail. Human HDAC1, the first HDA to be identified, was found to be homologous to yeast Rpd3 (Taunton *et al.*, 1996). Like Gcn5p, Rpd3 was originally identified as being a regulator of transcription. Subsequently, other HDAs have been identified (reviewed in Ng and Bird, 2000) . An important feature of HDAs is that they are targeted to promoters by associating with transcriptional co-repressors (section 1.4.2.3). Therefore, deacetylation completes the link between transcription and the acetylation state of the chromatin.

(c) Mechanisms for how acetylation affects transcription

Although it has been clearly demonstrated that histone acetylation and deacetylation have important roles in transcriptional regulation, the mechanisms by which these reactions regulate transcription are unknown. One model proposes that histone acetylation regulates the extent of chromatin compaction and folding (figure 1.6). For example, chromatin fibres reconstituted *in vitro* with hyperacetylated core histones are extended under physiological conditions. One reason for this observation is that the histone tails are known to interact strongly with linker DNA (section 1.2.1) and this interaction is inhibited by acetylation of the tails. Support for this suggestion has come from yeast genetic studies. Such studies have shown that substitution of appropriate lysines with uncharged residues to mimic acetylation on H3 results in chromatin decompaction and gene activation (Mann and Grunstien, 1992). In addition, a functional link has been demonstrated between histone

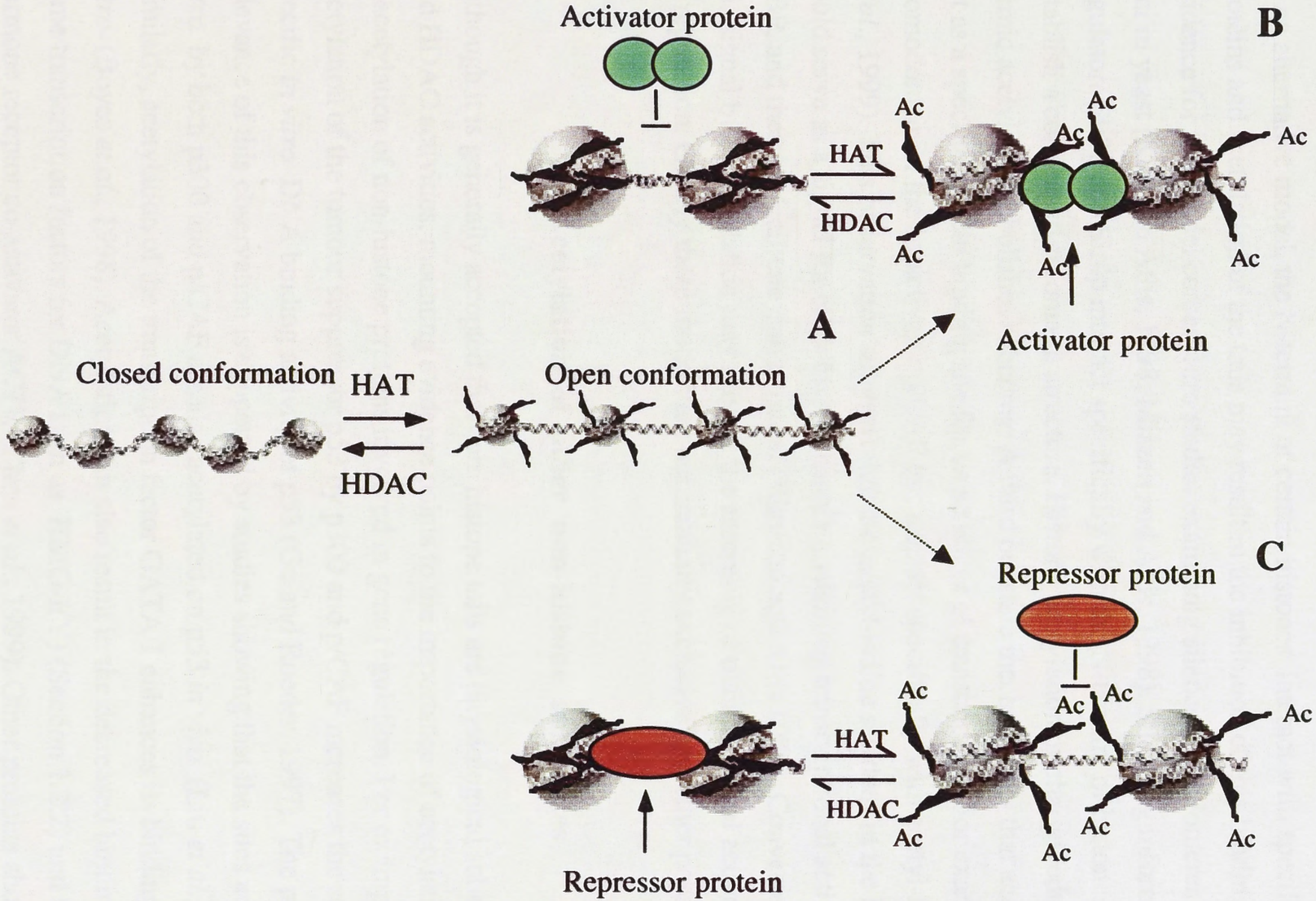


Figure 1.6: Schematic depiction of how histone acetylation may affect gene transcription. Histone acetylation may affect transcription in at least 3 ways. **A**. Histone acetylation neutralises the positive charge of the tails decreasing their affinity for DNA and may therefore, destabilise the structure of the nucleosome. **B**. The acetylated lysine on the histone tail may be a specific protein binding site for activator molecules. **C.** Acetylation of the histone tails may inhibit the binding of certain repressor molecules that would otherwise bind to the promoter and repress transcription.

acetylation and polyamines (Pollard *et al.*, 1999). Polyamines promote the oligomerization of nucleosome arrays *in vitro* but hyperacetylation inhibits the oligomerisation process. Such inhibition of polyamine interaction may result in a open chromatin structure that is compatible with high level transcription.

In an alternative model, the N-termini of certain histones interact with specific repressor proteins and acetylation of the tails may result in the inhibition of this binding. Some evidence for this model comes from studies examining silencing at telomeres and mating loci in yeast (Kuo and Allis, 1998; Mizzen and Allis, 1998). Silencing information regulators Sir3p and Sir4p interact specifically with the N-termini of histones H3 and H4 to establish a condensed chromatin structure. Histone acetylation or charge reduction, to mimic acetylation, abolishes silencing. A third possible mechanism is that acetylated lysines act as a specific protein binding site for an activator of transcription. For example the bromodomain of the co-activator p/CAF can interact specifically with acetyl-lysine (Ornaghi *et al.*, 1999). This observation suggests that the acetyl-lysine residues in the histone tails could serve as a signal for other bromodomain containing transcriptional activators such as CBP, and thereby facilitate transcription (Winston and Allis 1999). Conversely, removal of this signal by deacetylation may impede the assembly of transcriptional activators resulting in repression. Clearly these models are not mutually exclusive and other possibilities exist.

(d) Acetylation of other non-histone substrates

Although it is generally accepted that core histone tails are physiological substrates for HAT and HDAC activities, mounting evidence points to the importance of acetylation and deacetylation of non-histone proteins involved in gene regulation. For example, the acetylation of the tumour suppressor p53 by p300 and p/CAF increases the sequence specific *in vitro* DNA binding activity of p53 (Gu and Roeder, 1997). The physiological relevance of this observation is supported by studies showing that the sites acetylated *in vitro* by both p300 and p/CAF are also acetylated on p53 *in vivo* (Liu *et al.*, 1999). Similarly, acetylation of the transcription factor GATA 1 enhances its binding to DNA *in vitro* (Boyes *et al.*, 1998). Acetylation can also result in the decreased binding affinity of some transcription factors for DNA such as HMG-I(Y) (Section 1.2.2) and the nuclear hormone receptor co-activator ACTR (Chen *et al.*, 1999). Other proteins shown to be acetylated by HATs are EKLF, TFIIEβ and TFIIF(RAP30/74) (Imhof *et al.*, 1997) and this list is also growing.

(e) Other modifications

In addition to acetylation, other post-translational modifications of histone tails include phosphorylation, methylation, ubiquitination and ADP-ribosylation. Like acetylation, these modifications may also have important roles in transcriptional regulation. For example several kinases have been identified that phosphorylate serine 10 on histone H3 such as MSK1 (Thomson *et al.*, 1999) and a recently identified kinase, associated with dosage compensation in *Drosophila* (Jin *et al.*, 1999). Interestingly, mutations in a third histone kinase Rsk-2, are associated with Coffin-Lowry syndrome in humans (Sassone-Corsi *et al.*, 1999). Transcriptional activation in response to stimuli is altered in Coffin-Lowry cells, suggesting a direct role for H3 phosphorylation in regulating gene transcription.
A co-factor possessing histone methylation activity termed CARM1 (Chen *et al.*, 1999), which is required for ligand-dependent transcriptional activation, also suggest that methylation is involved transcriptional regulation.

1.4.2.3 Co-factors: Linking remodelling machines and acetylation to transcription

Co-factors have traditionally been defined as factors that do not bind to DNA, but are involved in transcription control by bridging sequence specific transcription factors with the basal transcriptional machinery. Co-factors include TAFs in TFIID, other members of the basal transcriptional machinery and gene specific factors such as CBP and p/CAF. Two important findings have revolutionised the way in which co-factors are thought to function in transcriptional control: the identification of co-factors that possess HAT and HDAC activity and the identification of co-factors in multi-subunit complexes that are involved with chromatin remodelling and acetylation. Furthermore, co-factors are thought to target chromatin remodelling and acetylation activities to the promoter regions of specific genes.

(a) Linking co-factor activity and acetylation

p300/CBP is a primary example of the integration between traditional co-factor activities and acetylation activities. p300/CBP is a global co-activator of transcription that is targeted by a wide range of sequence specific transcription factors including nuclear receptors, CREB, MyoD and Pit-1 to modulate transcription (Janknecht and Hunter, 1996; Goodman and Smolik, 2000). p300/CBP also possess high levels of HAT activity (Bannister and

Kouzarides, 1996; Ogryzko, *et al.*, 1996), which has been shown in some cases to contribute to transcriptional activation (Martinez-Balbas *et al.*, 1998; Kraus and Kadonaga 1998). A schematic diagram depicting the major structural features and protein interaction sites on CBP is shown in figure 1.7. Once recruited to the promoter, CBP is thought to activate transcription by acetylation of histone or non-histone substrates. However there appears to be a higher level of complexity in co-activation by CBP. In addition CBP recruits another co-factor with HAT activity: p/CAF, which interacts with many of the same sequence-specific transcription factors to form a multimeric complex at enhancers. Interestingly, although both CBP and p/CAF are present at promoters, their acetylation activities have gene specific effects. For example, Pit-1 dependent transcription requires CBP HAT activity when activated by cyclic AMP, whereas it requires p/CAF HAT activity when activated by growth factors (Xu *et al.*, 1998). Such studies show that sequence specific transcription factors act to target co-factor HAT activities to specific genes and that this targeting can have sequence specific effects on transcription.

(b) Linking acetylation machines to genes by co-factors

Recent data has demonstrated that histone acetylating co-factors are present as subunits of large complexes which also contain many additional proteins (Brown *et al.*, 2000). An example of such a complex is yeast SAGA (Spt-Ada-Gcn5-acetyltransferase), which preferentially acetylates H3 (Grant *et al.*, 1997; Roberts and Winston, 1997). SAGA contains at least 14 subunits, which can be divided into 4 classes. (a) an acetyl transferase activity (Gcn5p), (b) the Ada proteins (Ada1p, Ada2p, Ada3p, and Ada5p/Spt20p) which are a subset of the pol II TAFs. (c) The SPT proteins (Spt3p, Spt7p, Spt8p and Spt20p/Ada5p), that are proposed to play a role in TBP function, and (d) A subset of the TBP associated TAFs (TAFII20/15, TAFII25/23, TAFII60, TAFII68/61 and TAFII90). Although Gcn5p is thought to be responsible for the acetylation activities of SAGA, the other co-factors confer on Gcn5p the ability to acetylate an expanded set of lysine residues of H3 (Grant *et al.*, 1999). Furthermore, the additional co-factors are also thought to be involved in targeting SAGA acetylation activities to specific genes as the SAGA complex interacts with TBP and acidic transcriptional activators (Utley *et al.*, 1998; Sterner *et al*, 1999). This observation suggests the SAGA complex combines the activities of acetylation and traditional adaptors.

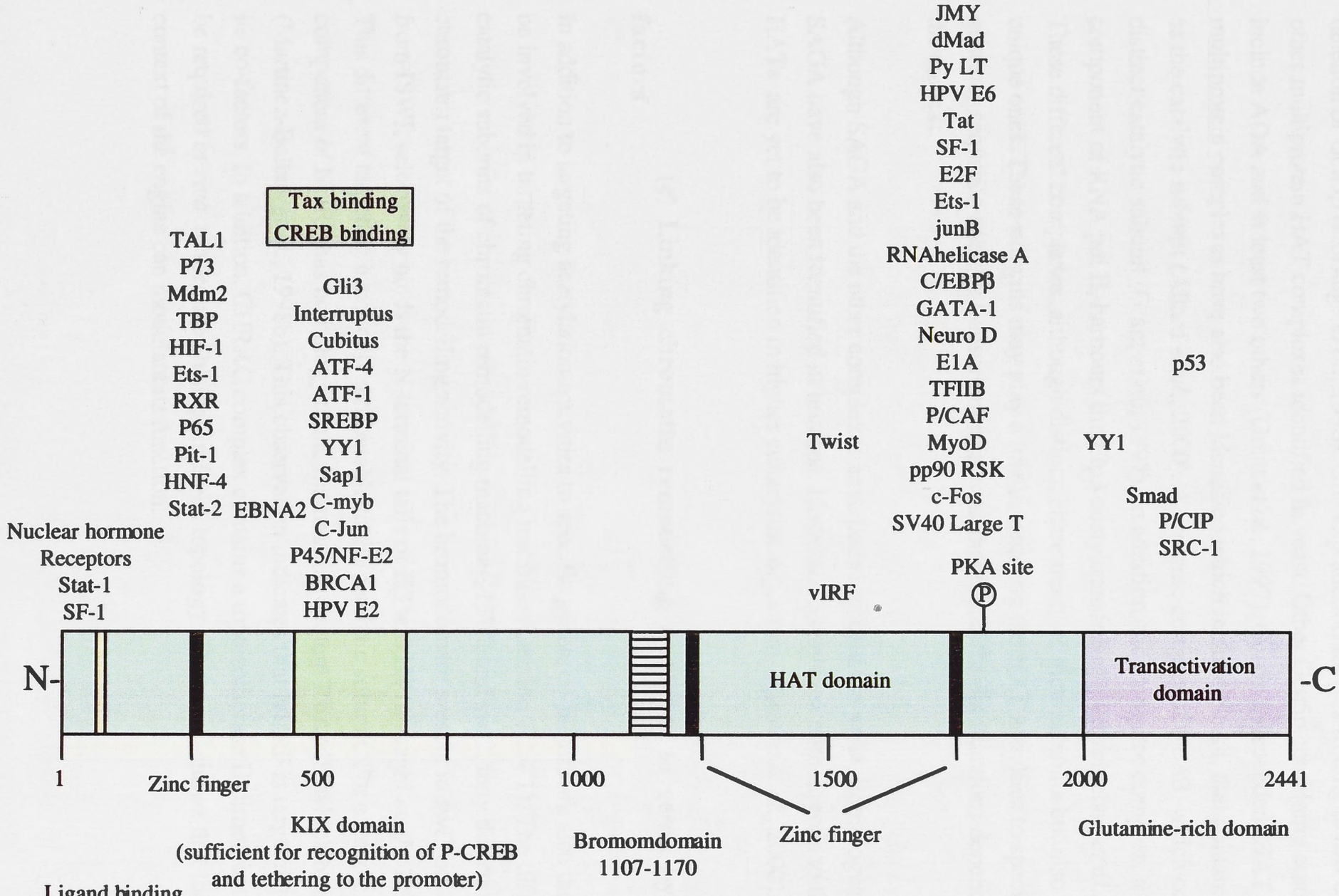


Figure 1.7: Domain structure and protein binding domains of CBP. Schematic diagram representing the major structural features (below the protein) and the binding domains of some of the known CBP interacting proteins (above the protein). The Tax and CREB binding domains are highlighted. Figure sourced from Goodman and Smolik (2000).

Interestingly, a genome wide analysis of SAGA function using high density arrays demonstrated that only about 5% of yeast genes show a strong decrease in expression upon deletion of Gcn5p (Holstege *et al.*, 1998). One explanation for this finding is that there are other multiprotein HAT complexes identified in yeast. Other Gcn5p containing complexes include ADA and at least two others (Grant *et al.*, 1997). Gcn5p independent HAT multiprotein complexes have also been identified which include NuA4, that contains Esa1p as the catalytic subunit (Allard *et al.*, 2000). A separate complex is NuA3, which contains a distinct catalytic subunit (Grant *et al.*, 1997). In addition, the elongator complex, a major component of RNA pol II, harbours the Elp3 acetyltransferase (Wittschieben *et al.*, 1999). These different complexes, although distinct, share many of their subunits but also contain unique ones. These subunits may play a role in targeting the HAT activities to specific genes or sequence specific transcription factors, thereby, different functions depend upon the context.

Although SAGA and the other complexes have been identified in yeast, homologues of SAGA have also been identified in humans. However, complexes homologous to the other HATs are yet to be identified in higher eukaryotic organisms (Brown *et al.*, 2000).

(c) Linking chromatin remodelling machines to genes by co-factors

In addition to targeting acetylation activities to specific genes, co-factors are also thought to be involved in targeting chromatin remodelling machines (section 1.2.4.1). The different catalytic subunits of chromatin remodelling machines, ISWI and swi2, may determine the chromatin target of the remodelling activity. The bromodomain present in swi2 but absent from ISWI, selectively binds the N-terminal tails of H3 and H4 (Ornaghi *et al.*, 1999). This different targeting is not only attributable to the catalytic subunit. For example, a component of NURF has homology to the mammalian protein RbAp48, which binds to H4 (Martinez-Balbas *et al.*, 1998b). This observation indicates that NURF is targeted to H4 by its co-factors. In addition, CHRAC complex contains a topoisomerase II dimer that could be required *in vivo* to mediate changes in DNA topology. This work shows that the context of the engine can modulate its function.

(d) Linking chromatin remodelling machines to acetylation and co-factors

ATP dependent chromatin remodelling machines and acetylation enzymes are thought of as separate classes of enzymes. However, recent evidence suggests that both activities may be present in large multisubunit complexes. One example of such a complex is the *Drosophila* chromatin remodeller NURD (nucleosome remodelling and histone deacetylation). NURD has been shown to contain ATP dependent remodelling activities through a protein called Mi2 (Xue *et al.*, 1998). In addition, NURD contains the subunits HDAC1 and HDAC2 that were identified as having histone deacetylation activity (Xue *et al.*, 1998). Similar work by Wade and Wolffe (Wade *et al.*, 1998) has shown that a multi subunit complex in *Xenopus* (termed Mi-2 complex) possesses ATPase activity in the Mi-2 subunit and histone deacetylase activity through multiple subunits including a Rpd3-like deacetylase. Similar complexes have also been identified in humans (Zhang *et al.*, 1998; Tong *et al.*, 1998). In one proposed model, it is thought that these factors use chromatin remodelling activities to make the nucleosomes accessible to deacetylation.

Complexes such as NURD and Mi-2 complex not only couple deacetylation activities to chromatin remodelling, but also link these two processes to co-factor mediated targeting. Both the NURD and Mi-2 complexes contain additional subunits which include a methylated DNA recognising protein termed MBD3 (Wade *et al.*, 1999; Zang *et al.*, 1999). This co-factor is thought to target the Mi-2 complex to methylated DNA where it can participate in silencing by chromatin remodelling and deacetylation. However the MBD2/3 proteins recognise methylated DNA non-specifically. Further refinement of the targeting ability of the NURD complex is brought about by its recruitment of sequence specific binding proteins. For example, the sequence specific transcription factor Ikaros and Aiolos has been shown to recruit NURD through its Mi2 subunit to heterochromatin regions (Kim *et al.*, 1999). Thus the Mi2 containing complexes couple DNA methylation to chromatin remodelling and histone acetylation.

1.4.3 Histone H1 and transcriptional regulation

In addition to core histones, the linker histone H1 is also thought to have a role in the regulation of gene expression. However, the role of histone H1 in the regulation of transcription has proved to be a contentious issue in the literature. Early studies suggested

that H1 acted as a non-specific inhibitor of transcription (Croston *et al.*, 1991; Laybourn and Kadanoga, 1991). However more recent evidence has indicated that, just as for the core histones, H1 regulates the expression of specific genes. For example in *Xenopus*, increased expression in embryos specifically repressed the expression of 5S RNA genes but not the transcripts of other pol III genes (Bouvet *et al.*, 1994). Furthermore, studies in *Tetrahymena* have showed that H1 is not essential for survival (Shen *et al.*, 1995). In addition, these investigators demonstrated that H1 does not affect global transcription in *Tetrahymena*, but the expression of specific genes (Chen and Gorovsky, 1996). These studies suggest that H1 may interact in some way with the transcriptional machinery of different genes and thus have a regulatory role in gene expression. One mechanism by which H1 can regulate transcription is by its phosphorylation. Lee and Archer, (1998) have demonstrated that HI at the transcriptionally inactive mouse Mammary Tumour Virus (MMTV) promoter is dephosphorylated in response to glucocorticoids. Whereas removal of glucocorticoids results in the phosphorylation of H1 and the reaquisition of transcription competence. Therefore, to understand the full impact of chromatin on transcriptional regulation of specific genes, the role of H1 must also be taken into account.

1.5 Retrovirus transcription as a model for factor mediated chromatin remodelling

Chromatin compaction of DNA in eukaryotes establishes a unique situation for Retroviruses. An essential aspect of the retrovirus life cycle is the integration of the provirus into the host cell chromosome. Unlike viruses that do not integrate, retroviruses must have evolved and conserved the ability to activate transcription from a repressive chromatin environment. The Human T-cell leukemia virus types I and II (HTLV-I and II), The Human Immunodeficiency Virus types I and 2 (HIV-1 and HIV-2) and the human foamy virus (HFV) are the only known human retroviruses.

HIV-1 is the most comprehensively studied paradigm for retroviral transcription. Work from various laboratories has shown that chromatin is a major contributory factor in the regulation of HIV-1 provirus expression (Verdin *et al.*, 1993; Pazin *et al.*, 1996; Widlak *et al.*, 1997). Elegant work by Verdin *et al.* (1993) has shown that the 5' integrated HIV-1 DNA contains an array of precisely positioned nucleosomes which define two large nucleosome free areas. In addition, a nucleosome termed nuc-1 was found immediately after the transcription initiation site. This work indicates that upon integration, HIV-1 creates its own chromatin environment. The creation of this specific environment may be

the result of nucleosome positioning sequences found within the DNA sequence or the effect of cellular or viral *trans*-acting factors.

Importantly, transcription induction of the integrated HIV-1 DNA is accompanied by specific disruption of nuc-1 independently of transcription and replication (Verdin *et al.*, 1993). This result indicates the presence of mechanisms for chromatin remodelling. One of these mechanisms may involve acetylation as, addition of the deacetylase inhibitor TSA, strongly induces HIV-1 transcription on chromatin templates *in vivo* (Sheridan *et al.*, 1997).

HTLV-I was the first human retrovirus discovered, although unlike HIV-1, the chromatin structure of the integrated provirus has yet to be characterised. However, treatment of chronically infected cells with the deacetylase inhibitor sodium butyrate results in the induction of HTLV-I gene expression (Lin *et al.*, 1998). This data suggests that the acetylation of chromatin may have a role in the regulation of HTLV-I viral gene expression.

At the conception of this research study it was established in the literature that the viral transactivator, Tax, interacted with the cellular protein CREB on the HTLV-I LTR. This interaction was thought to bring about the recruitment of the co-factor CBP (Kwok *et al.*, 1996) (section 1.2.2.3a). Importantly, CBP had just been identified as not only a co-activator of transcription but as a histone acetyltransferase. The interactions described, have only been investigated on naked DNA templates. However, chromatin compaction clearly plays a vital role in the transcription of retroviral DNA. Therefore, this research study exploits the HTLV-I promoter, and the factors that interact with it, as a model to define mechanisms that control chromatin de-repression. In particular The HTLV-I system has novel potential to investigate the targeting of the newly identified histone acetyltransferase CBP. Understanding the interaction between cellular factors and chromatin on the HTLV-I LTR may also contribute to a greater understanding of the mechanisms involved in viral pathogenesis and transformation.

1.6 HTLV-I

The Human T-Cell Leukemia Virus type I (HTLV-I) was the first retrovirus to be discovered and isolated (Yodi and Uchiyama, 1992). The virus appears to be the cause of at least two diseases: Adult T-Cell Leukemia (ATL) and Tropical Spastic Paraparesis (TSP), also known as HTLV-I associated myelopathy (HAM) (Sodroski, 1992; Wong-

Staal and Gallo, 1985). ATL is characterised by abnormal proliferation of functionally impaired T helper (Th) lymphocytes (Wong-Staal and Gallo, 1985). The disease has a long latency period where the time between infection and the clinical manifestations of the disease range between ten and thirty tears. Like other retroviruses that exhibit latency periods, the reason for the latency and the mechanisms involved are unclear.

HTLV-I infection is endemic in south-western Japan, in the Caribbean islands and in central Africa (Yodi and Uchiyama, 1992). In addition, seropositivity has also been reported in Australian Aboriginals (Bastain, 1992). However, it has been estimated that less than 5% of HTLV-I carriers will develop ATL. Unfortunately the prognosis of acute leukemia is very poor with a 6-month to 2-year survival time (Smith and Greene, 1991; Bastain, 1992; Yodi and Uchiyama, 1992).

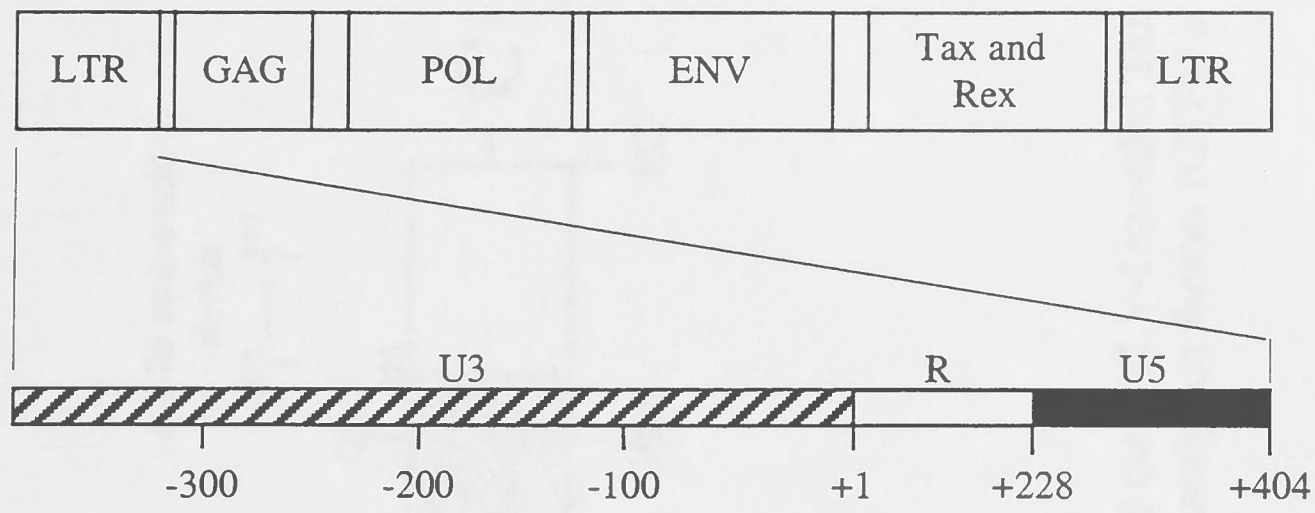
1.7 Tax

The genome of HTLV-I has a similar structure to that of other retroviruses in that the genome contains the typical retroviral genes: *gag*, *pol* and *env* (Figure 1.8). These genes encode the nuclear core proteins, non-structural proteins such as reverse transcriptase and the envelope proteins, respectively (Pavlakis *et al.*, 1992). Flanking the genome on both the 5' and 3' ends are the tandem long terminal repeats (LTR) which provide sequence elements that regulate viral gene expression.

The 3' region of the genome encodes a region termed pX, which is unique to the HTLV family. The pX region encodes at least two non-structural proteins: a 40 KDa protein Tax, and a 27 KDa protein Rex. Tax and Rex are encoded by the same doubly spliced RNA but from different open reading frames (Feuer and Chen, 1992). Rex is thought to facilitate the transport of viral genes into the cytoplasm allowing their translation (Smith and Greene, 1991). Tax is the major regulatory protein involved in increasing viral gene expression and its interaction with cellular genes has been proposed to cause transformation of the host cell. A diagram of the major functional domains of Tax and the proteins that interact with it is shown in figure 1.9. Tax is vital to the life cycle of HTLV-I, as efficient transcription is strongly dependent on this protein (Chen *et al.*, 1995). Unlike conventional oncogenes, Tax does not bind to DNA directly but stimulates transcription by interaction with cellular transcription factors. Tax has a dual function leading to the progression of disease:

a. Interaction with cellular genes resulting in transformation of T cells
b. Interaction with the HTLV-I LTR triggering viral transcription

A



B

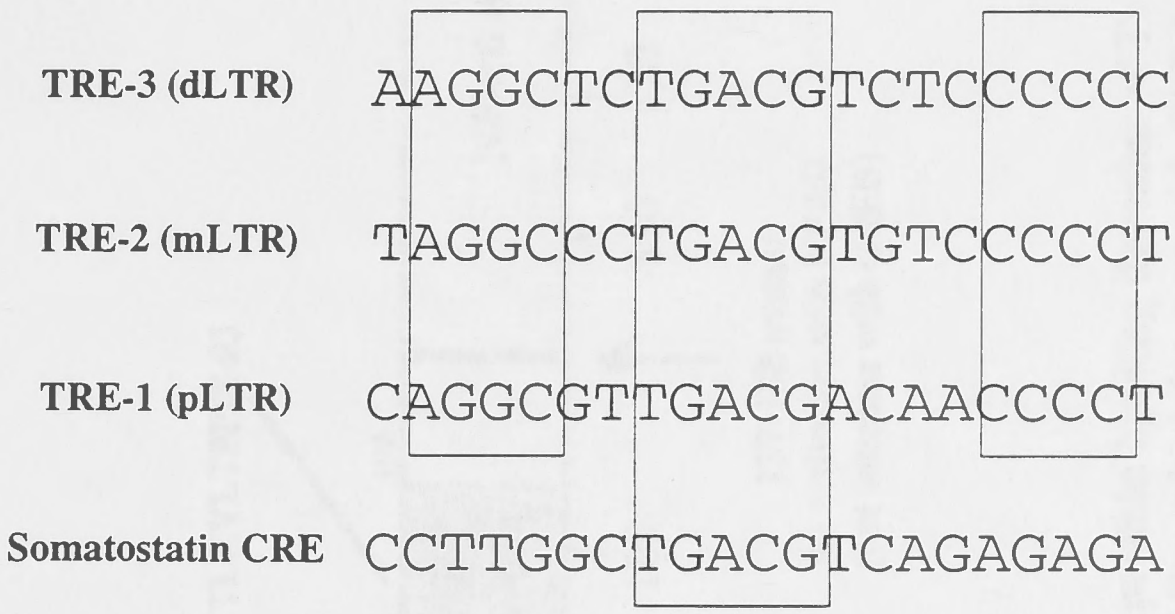


Figure 1.8: Structure of the HTLV-I LTR. A. Schematic diagram representing the structure of the HTLV-I LTR in the context of the viral genome. The genomic structures within the 9 Kb genome are indicated by the labelled boxes. The core proteins which surround the RNA genome (p19, p24 and p15) are encoded by the *gag* gene. The envelope glycoproteins (gp46 and gp21) are encoded by the *env* gene and are positioned within the host cell derived lipid membrane. Reverse transcriptase and other replicative enzymes are encoded by the *pol* gene. A unique region to the HTLV-I family encodes the Tax and Rex proteins. The 21-bp repeat elements are critical for the regulation of viral gene expression and are contained within the 3' and 5' LTRs at positions -251 to -231, -203 to -183 and -103 to -83 relative to the start site of transcription. **B.** Sequence comparison between the three 21 bp repeat elements and the cellular CRE element from the somatostatin gene. The regions of strict conservation are indicated by a box. TRE-1 is located proximal to the TATA box (pLTR), TRE-2 is the middle element (mLTR) and TRE-3 is the distal element (dLTR).

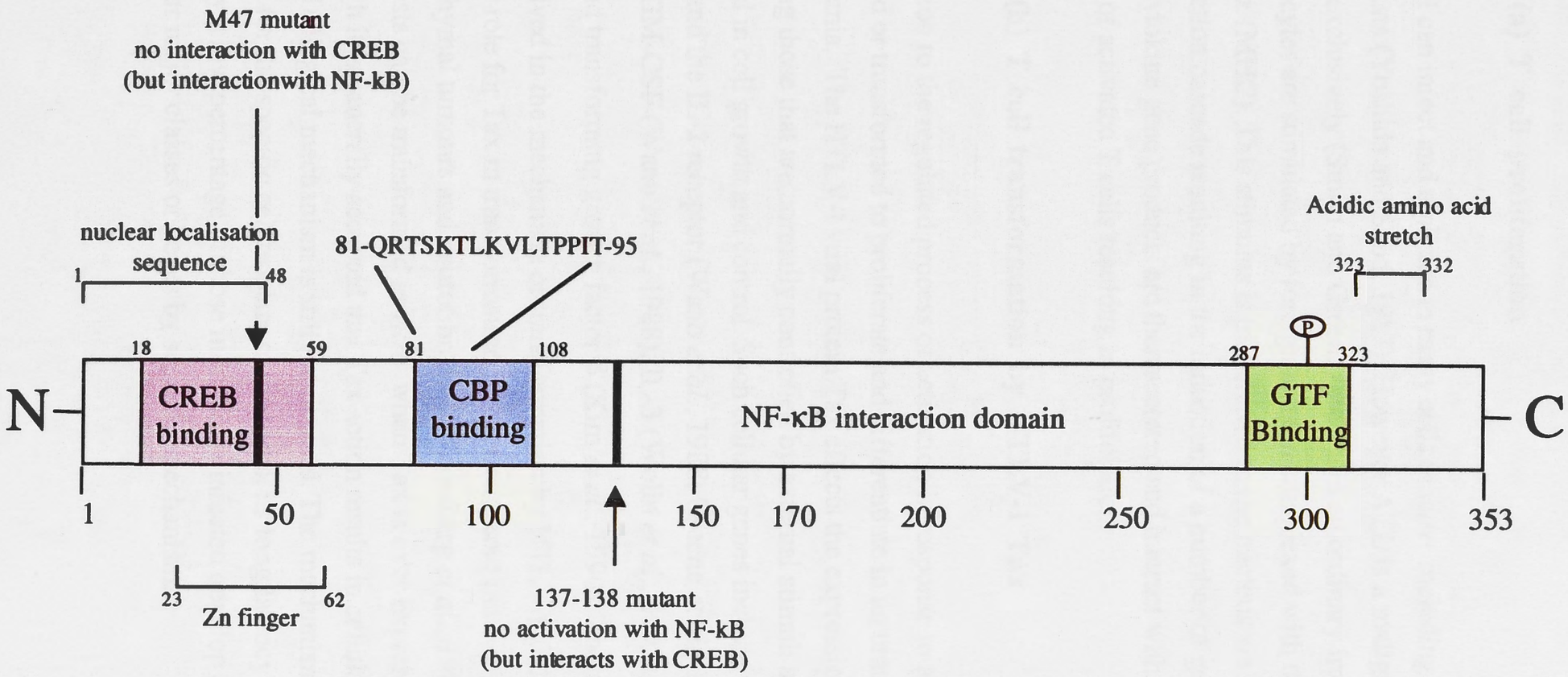


Figure 1.9: Domain structure and protein binding domains of Tax. The major structural features of the HTLV-I encoded Tax protein are indicated. The regions of Tax which interact with CREB, CBP, NF-κB and the general transcription factors (GTFs) is also indicated. The positions of two highly characterised Tax mutants are shown by a black line.

1.7.1 Interaction of Tax with cellular genes

(a) T cell proliferation

HTLV-I can infect and replicate in many cells *in vitro* including B cells, epithelial cells and fibroblasts (Yoshida and Seiki, 1987). However ATL is a malignancy of Th lymphocytes almost exclusively (Smith and Greene, 1991). In an ordinary immune response, resting lymphocytes are stimulated by foreign antigen complexed with the major histocompatability complex (MHC). This stimulus is transmitted to the nucleus via a complex signal transduction cascade resulting in the induction of a number of genes including cytokines. These cytokine gene products are then secreted and interact with receptors on the cell surface of activated T cells resulting in proliferation.

(b) T cell transformation by HTLV-I Tax

In addition to the regulated process of activation in response to antigen, T cells may also be activated or transformed to proliferate and differentiate in an unregulated manner resulting in leukemia. The HTLV-I viral protein Tax effects the expression of cellular genes including those that are normally controlled by external stimuli and whose products are involved in cell growth and control. Such cellular genes include the cytokines interleukin (IL) -2 and the IL-2 receptor (Wano *et al.*, 1988; Greene *et al.*, 1986 and Siekevitz *et al.*, 1987), GM-CSF (Wano *et al.*, 1988), IL-3 (Wolin *et al.*, 1993), IL-6 (Muraoka *et al.*, 1993 and transforming growth factor β (Kim *et al.*, 1990). Therefore Tax is speculated to be involved in the mechanism of transformation by HTLV-I. Other evidence supporting a primary role for Tax in transformation is that transgenic mice expressing Tax develop mesenchymal tumours and neurofibromas (Nerenberg *et al.*, 1987). In addition, rat fibroblasts can be transformed *in vitro* when Tax is over expressed (Tanaka *et al.*, 1990). Although it is generally accepted that Tax action results in cellular transformation, the precise biological mechanism is still not defined. The mechanism of transformation must account for the specific cell tropism of the virus, its long latency period and the observation that only a low percentage of those individuals infected develop disease. Tax can interact with four major classes of genes by separate mechanisms.

1) The CArG box genes, which includes early response genes such as *c-fos*, *c-jun*, *fra-1*, *erg1* and *erg2*: The CArG box binds a dimer known as Serum Response Factor (SRF). Phosphorylation of SRF alters its DNA binding activity or activation potential (Edwards *et al.*, 1994). It has been shown that Tax can interact with SRF to cause the activation of *c-fos*, *erg1* and *erg2* (Fujii *et al.*, 1992).

2) NF-κB responsive genes including the cytokines IL-2, IL-2 receptor and the HIV LTR: The NF-κB family of transcription factors are tethered in the cytoplasm by high affinity binding with inhibitors known a IκB (Zabel and Baeuerle, 1990). Tax is thought to induce phosphorylation, ubiquitination and proteolic breakdown of IκB leading to the elimination of IκB from Tax expressing cells (Beraud and Greene 1996; Suzuki *et al.*, 1993). The mechanism involved in Tax mediated phosphorylation of IκB is not yet understood although it is thought that Tax activates IKK2, a recently identified IκB kinase (Yin *et al.*, 1988).

3) Basic helix-loop-helix (bHLH) responsive genes such as β-polymerase and MyoD: In contrast to the activation of the above cellular genes, Tax represses the expression of β-polymerase by interaction with the basic helix-loop-helix (bHLH) transcription factor. This repression is thought to result from competition between Tax and bHLH transcription factors for a binding site on p300 (Riou *et al.*, 2000).

4) The fourth class of Tax responsive gene is not a cellular gene but the viral LTR. Interaction between Tax and the viral LTR is the central area of investigation of this research work and will be discussed in detail.

1.8 Interaction of Tax with the HTLV-I LTR

As previously described in section 1.7, Tax stimulates transcription of HTLV-I through interaction with host cell transcription factors instead of through direct binding to the HTLV-I promoter. The cellular proteins that interact with the HTLV-1 LTR can be divided into two groups. Firstly proteins that are not responsive to Tax and proteins which are Tax responsive.

1.8.1 Non-Tax responsive proteins

Several proteins have been shown to bind to the HTLV-I viral LTR including Elf-1, c-Myb, Ets-1, Ets-2 and AP-1. However it has been demonstrated that these protein are not responsive to Tax (Fujii *et al.*, 1995; Jeang *et al.*, 1991). These cellular factors may be involved in HTLV-I transcription when transcription is independent of Tax, For example when transcription is initiated in newly infected T-cells or in the reactivation process from latent infection.

1.8.2 Tax responsive proteins

The Tax responsive transcriptional control region of HTLV-I contains three imperfect 21-bp repeat elements within the U3 region of the virus (figure 1.8) each containing the motif TGACGT immediately flanked by a short run of GC-rich nucleotides. The three 21bp repeats are known collectively as the Tax responsive element (TRE). The TGACGT motif is highly homologous to *cis* elements (TGACGTCA) on cellular genes which confer cAMP inducibility to a wide variety of cellular genes including c-Fos, α-choriogonatropin gene and the vasoactive intestinal peptide gene (Montminy, 1997) (figure 1.10), and is termed cAMP-responsive element (CRE). There is a large body of evidence that suggests that the 21 bp repeats are the primary targets for transcriptional activation by Tax. For example the repeats are the only conserved element in the U3 region between HTLV-I and HTLV-II other than the TATA box, the polyadenylation site and a region around the cap site (Paskalis *et al.*, 1986). Since both viral LTRs are Tax-2 responsive, this observation suggests that the 21pb repeat is the region of interaction between Tax-2 and the viral LTR. Experimental work has used deletion and saturation metagenesis of the HTLV-1 LTR and chemically synthesised oligonucleotides to construct hybrid promoter constructs that show that the 21bp repeat is directly responsive to Tax (Paskalis *et al.,* 1986; Brady *et al.*, 1987; Fujisawa *et al.*, 1989; Giam and Xu, 1989).

Several lines of evidence suggest that members of the ATF/CREB family of transcription factors are the principal TRE binding proteins. Although many cellular proteins have been identified as TRE binding proteins (Jeang *et al.*, 1988; Tan *et al.*, 1989; Yoshimura *et al.*, 1990; Montagne *et al.*, 1990; Xu *et al.*, 1990; Nyborg and Dynan *et al.*, 1990), molecular cloning of cDNAs encoding these proteins and DNA affinity chromatography using

Somatostatin	TTGGC**TGACGTCA**GAGAGAGAG	(-32)
PEPCK	GCCCC**TTACGTCA**GAGGCGAGC	(-74)
VIP	TACTG**TGACGTC**TTTCAGAGCA	(-60)
PTH	GGGAGT**GACGTCA**TCT	(-65)
Enkephalin	GGGCCTG**CGTCA**GC	(-87)
αCG	AAAAT**TGACGTCA**TGG	(-113)
fos	CCCAG**TGACGT**AGGA	(-57)
CMV	CCCAT**TGACGTCA**AT	(-124)
BLV	GACAGA**GACGTCA**GCTG	(-144)
HTLV-I	GGCCC**TGACGTC**CCTCC	(-162)
Secretogranin	GCCGG**TGACGTCA**GCGT	(-74)
Chromogranin II	CTCCG**TGACGTCA**GCGT	(-108)
Synapsin I	CGCGC**TGACGTCA**CTCG	(-166)
LDH	CACTC**TGACGTCA**GCGC	(-48)
Aromatase	TATGC**ACGTCA**CCCA	(-161)
Fibronectin	CCC**TGACGTCA**CCC	(-170)
αA Crystallin	ACCAGACT**GTCA**TCCC	(-148)

Figure 1.10: Sequence alignment of cAMP responsive elements.
Promoter sequences for various genes are shown with the 3'-nucleotide position indicated in parentheses on the right. Homologous sequences are indicated in bold. (Source: Montminy, 1997).

immobilised 21bp repeats has revealed that many of these proteins represent members of the CREB/ATF family of DNA binding proteins (Yoshimura *et al.*, 1990; Tsujimoto *et al.*, 1991; Beimling and Moelling, 1992). In addition, genetic experiments using reporter genes in *Saccharomyces cerevisiae* have identified TRE binding proteins which are members of the CREB/ATF family (Bantignies *et al.*, 1996; Shnyreva and Munder, 1996).

The importance of the CREB/ATF pathway in Tax mediated transformation of rat fibroblast cells has been shown by Smith and Greene (1991). These investigators have used mutant versions of Tax that functionally segregate the cAMP and NF-κB pathways of trasactivation (figure 1.9). The NF-κB mutant, which retained the ability to transactivate the cAMP pathway, caused transformation of rat fibroblast cells. However, the cAMP mutant which retained the ability transactivate the NF-κB pathway appeared phenotypically normal. These results suggest that the ATF/CREB pathway plays a major role Tax-mediated cellular transformation.

1.8.2.1 The ATF/CREB family

Tax has been reported to interact with several members of the ATF/CREB family, including CREB, CREB-2, cAMP response element modulator (CREM), ATF-I, ATF-2 and ATF - 3. However it is now established in the literature that CREB is the principal Tax responsive T-cell protein that binds to the three 21-bp repeats and stimulates transcription *in vitro* (Franklin *et al.*, 1993; Brauweiler *et al.*, 1995; Shnyreva and Munder 1996). The present work investigates the mechanisms involved in CREB and Tax mediated transactivation of the HTLV-I-LTR. Thus, an overview of CREB function is presented below.

CREB is a member of the basic leucine zipper (bZIP) transcription factor family, which includes proteins such as Fos, Jun, ATF-2 and c-Myc. This group of proteins binds as dimers to DNA through a carboxy terminal basic region/leucine zipper motif. Although leucine zipper dimerisation domains have been thought to promote heterodimerization between other bZIP family members, CREB appears to bind DNA primarily as a homodimer (Dwarki *et al.*, 1990). A schematic diagram showing the functional domains of CREB is depicted in figure 1.11.

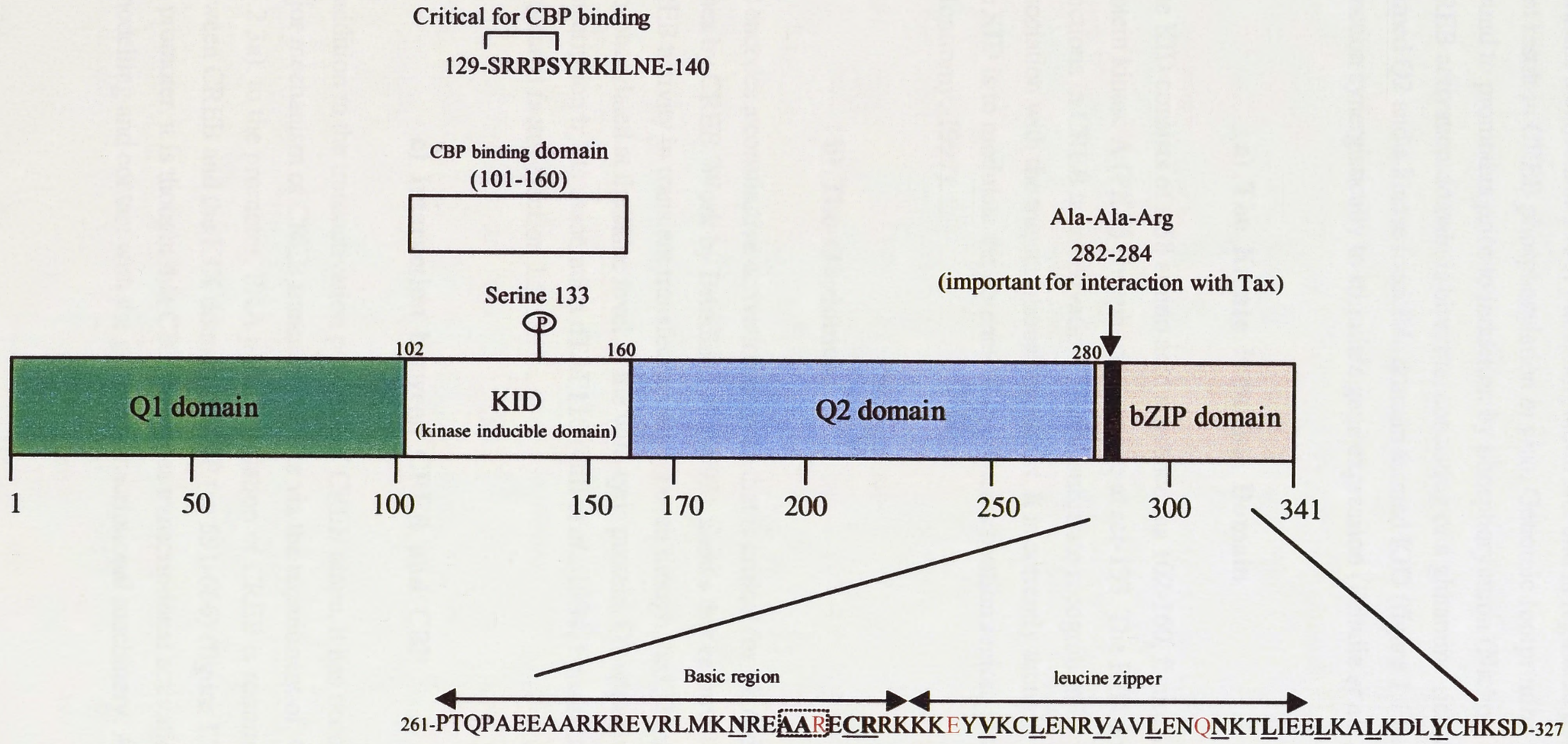


Figure 1.11 Domain structure and protein binding domains of CREB. The four major protein domains of CREB are indicated by the coloured boxes. The amino acids critical for binding of CBP are bracketed (Shih *et al.*, 1996). The amino acid sequence from 261 to 327 of the bZIP domain is shown below the protein. The Ala-Ala-Arg region important for interaction with Tax by Adya *et al.*, (1994) is indicated by a broken box. Regions important for Tax interaction by Tang *et al.*, (1998) are highlighted in red. The amino acids shown by Konig and Richmond, (1993) to contact DNA are in bold and underlined.

CREB is a transcription factor involved in the cAMP response pathway. This factor contains a consensus PK-A phosphorylation site and is regulated by extracellular signals that result in CREB phosphorylation *in vivo*. Genomic footprinting shows that CREB is bound to promoters prior to induction by phosphorylation (Nichols *et al.*, 1992). The CREB activation domain is bipartite, consisting of a glutamine-rich constitutive activator termed Q2 and a kinase inducible domain termed KID (figure 1.11). These two regions function synergistically to stimulate gene expression (Brindle *et al.*, 1993).

a) The Kinase Inducible Domain

The KID consists of a 58 amino acid sequence (aa 102-160, figure 1.11) that contains the protein kinase A (PKA) phosphorylation site at ser-133. The KID domain has two critical functions in CREB transactivation. Firstly, substrate recognition by PKA and secondly association with the transcriptional apparatus. It is currently thought that the major role of the KID is to modulate the potency of the other activation region, the Q2 domain (Montminy, 1997).

b) The Q2 domain

Q2 encodes a constitutive activation domain that is critical for PKA dependant induction of genes by CREB. Work by Brindle *et al.* (1993), shows that removal of Q2 abolishes CREB activity in transient transfection assays even though the Q2 mutant was phosphorylated at the same level as the wild-type protein. Q2 appears to stimulate transcription by its association dTAF110 (Gill *et al.*, 1994, Ferreri *et al.*, 1994), a TBP associated factor (section 1.3.1).

c) Interaction between CREB and CBP

In addition to the transactivation potential of CREB alone, it has become apparent that the major mechanism of CREB transactivation is via the recruitment of CBP (section 1.4.2.3a), to the promoter. PKA phosphorylation of CREB is required for association between CREB and the KIX domain of CBP (aa 591-666) (figure 1.7). Once tethered to the promoter, it is thought that CBP facilitates transcriptional activation through chromatin remodelling and contact with the general transcriptional machinery. A major aim of this

thesis is to examine this hypothesis with regard to HTLV-I transcription. Thus, these interactions will be further discussed in section 1.8.4.

1.8.3 Interaction between Tax and CREB

One way that Tax is thought to activate transcription from the HTLV-I promoter is by increasing the binding affinity of CREB for the TRE elements. However, a considerable amount of controversy exists in the literature with regard to the mechanism by which this occurs. Based on work in the absence of DNA, Wagner and Green, (1993) showed that Tax increased the amount of CREB bound to the TRE by directly enhancing in the formation of bZIP homodimers. These observations led Wanger and Green to propose that Tax affects the binding of CREB without stably associating with the CREB-DNA complex. Similarly, other studies have also shown that Tax does not stably bind to gel-retarded complexes containing CREB (Franklin *et al.,* 1993; Yin *et al.*, 1995a, 1995b; Armstrong *et al.*, 1993)

On the other hand, several groups have observed that Tax may be involved in a stable ternary complex with CREB and DNA as visualised by EMSA (Zhao and Giam, 1991, 1992; Suzuki *et al.*, 1993; Anderson and Dynan 1994 Perini *et al.*, 1995 Brauweiler *et al.*, 1995; Yin and Gaynor, 1996). Furthermore, CREB-Tax-DNA interactions have been shown *in vivo* by the two-hybrid and immobilised template assays (Yin *et al.,* 1995). Therefore, it appears that a Tax-CREB-DNA complex forms in solution but dissociates under standard native gel electrophoresis conditions. More recently, several lines of evidence including quantitative EMSA, equilibrium binding and dissociation kinetics indicate that the site of interaction of Tax with bZIP proteins, although not exclusively, is the basic-spacer region within the bZIP domain (Adya *et al.*, 1994; Baranger *et al.*, 1995; Perini *et al.*, 1995; Yin *et al.*, 1995) (figure 1.11). Work by these groups support a model in which Tax stabilises a distinct DNA-bound protein structure. Furthermore, Lundblad *et al.* (1998) have used fluorescence quenching to show that CREB dimerises in the absence of DNA and that Tax does not enhance dimerisation. Thus, the proposed mechanisms of Tax-enhanced binding of CREB to DNA and ternary complex formation are controversial but may involve an increase in CREB dimerization and/or stabilisation of the structure of CREBs bZIP domain.

There has been much recent work on defining the geometry of the Tax-CREB-DNA ternary complex as its precise stoichiometry remains unresolved. Lenzmeier *et al.*, (1998) have

used cross-linking experiments and stoichiometric quantitation in EMSA to provide evidence that only a single molecule of Tax is present in the ternary complex. However, work by other groups using various different experimental strategies have provided evidence that Tax exists as a homodimer in the ternary complex. (Jin and Jeang, 1997; Tie *et al.*, 1996 and Kimzey and Dynan, 1998). Solving the precise structure of the ternary complex will be crucial in the elucidation of the mechanism of HTLV-I transcriptional activation by Tax.

Another interesting aspect of the interaction between Tax, CREB and DNA is the specificity of Tax-CREB transcriptional activation to the TRE-1 21bp repeats on the HTLV-I LTR. For example, Tax-CREB (not phosphorylated) does not activate CREB binding or transcription from the somatostatin promoter (Fujisawa *et al.,* 1989; Yin *et al.*, 1995, 1996). The somatostatin promoter contains the CRE core sequence (figures 1.8 and 1.10) and can be transcriptionally activated by CREB (Montminy *et al.,* 1986). These results indicate that differences in DNA sequences between the 21bp repeat and the somatostatin CRE are critical for Tax stimulation. Detailed work by Paca-uccaralertkun *et al.* (1994), using *in vitro* selection for preferred Tax-CREB binding sites, demonstrated that the Tax-CREB complex exhibits greatly increased DNA recognition specificity and assembles preferentially on CRE motifs flanked by long runs of G (5') and/or C (3') residues as found in the HTLV-I LTR. Furthermore, Perini *et al.* (1995) have also demonstrated higher levels of activation by Tax-CREB on core elements that are flanked with GC rich sequences than core sequences which are not.

A novel approach to investigate the differences between the HTLV-I 21bp repeat and the somatostatin CRE has been the analysis of DNA bending. Phasing analysis conducted by Yin *et al.* (1995) has shown that binding of CREB causes directed DNA bending of the HTLV-1 21bp repeat and the somatostatin CRE flanked by the 21bp repeat GC sequences. However, CREB did not induce DNA bending of the wildtype somatostatin CRE and Tax did not modify the CREB-induced DNA bending. Since CREB binding induces different DNA structural changes in the HTLV-I 21bp repeat to the somatostatin CRE, Yin *et al* (1996) have postulated that CREB protein adopts different conformations upon binding to different DNA binding sites. Further, that the interaction of Tax with CREB at the different binding sites, while not affecting DNA structure, may alter the CREB conformation to overcome a high energy barrier to CREB binding to the 21bp repeat. Results such as these indicate a major role for the GC rich flanking sequences contained in the 21bp repeats of

the HTLV-I LTR in the binding specificity and transactivation ability of the CREB-Tax complex, however the precise molecular mechanisms remains ill-defined.

More recently, the use of high resolution footprinting technology such as methidiumpropyl-EDTA iron(II) and 1,10-Phenanthroline-copper DNA footprinting has shown that Tax lengthens the CREB footprint into the GC rich flanking sequences on the HTLV-I LTR but has no effect on the somatostatin promoter (Lundblard *et al.*, 1998; Lenzmeier *et al.*, 1998). In addition, chromomycin A3, a minor groove-binding drug with GC selectivity, has been shown to specifically block the Tax-enhanced DNA binding activity of CREB and prevents formation of the ternary complex on the HTLV-I LTR (Lundblard *et al.*, 1998; Lenzmeier *et al.*, 1998). This body of evidence suggests that Tax directly interacts with the GC rich flanking DNA of the HTLV-I LTR. Therefore, another possible mechanism by which Tax activates HTLV-I transcription is by sequence specific stabilisation of CREB on the HTLV-I promoter by interaction with minor groove GC rich sequences. This mechanism may explain the exquisite DNA sequence specificity of Tax-mediated LTR transactivation.

The interactions between Tax, CREB and DNA discussed so far support a model in which Tax transactivates HTLV-I gene expression by increasing the number or the stability of CREB molecules bound to the viral promoter, leading to transcription of the viral DNA. However as outlined in section 1.8.2.1, transcriptional activity of CREB is largely dependent on phosphorylation by PKA. Thus, a model proposing that Tax increases the amount of CREB bound to the promoter incompletely explains the large increase in transcriptional activity caused by Tax. This concept has been illustrated by domain-swapping experiments where the bZIP DNA binding domain of CREB was replaced by the GAL4 DNA binding domain and used in conjunction with HTLV-I LTR-CAT promoter, whose 21bp repeats were substituted with the GAL4 binding site (Bodor *et al.*, 1995). In the presence of Tax, this chimeric protein activated the expression of the HTLV-I LTR CAT construct 9.6 fold even though the dimerization or stability of GAL4 is not affected by Tax. However, mutation of Serine-133 in the CREB-GAL4 protein diminished this Tax response. These results implicate an important role for phosphorylation of CREB in the Tax response and suggest a mechanism apart from, and/or in addition to the stabilisation of CREB-DNA complex. Bodor *et al.*, (1995) have suggested that such a mechanism may be an enhancement of interactions between CREB and CBP caused by Tax.

1.8.4 Interaction between Tax, CREB and CBP

It has been shown that Tax binds *in vitro* to CBP residues 451-682 (figure 1.7) and the corresponding portion of the p300 homologue p300 (566-663) (Kwok *et al.*, 1996). These regions have been previously characterised as containing the CREB binding domain and has been termed the 'KIX domain' (Parker *et al.*, 1996). Kwok *et al.* (1996) has further demonstrated, using an avidin-biotin complex assay, the existence of a quaternary complex consisting of non-phosphorylated CREB, Tax, CBP and the HTLV-I TRE. By contrast, Tax associated with the somatostatin CRE only in the presence of phosphorylated CREB and CBP. These results led Kwok *et al.* (1996) to propose a model in which CREB must be phosphorylated on the somatostatin CRE in order to recruit CBP in the presence of Tax. However, Tax interaction on the HTLV-I TRE, negates the need for CREB to be phosphorylated in order to form a complex with CBP. Thus Tax acts as a bridging protein between unphosphorylated CREB and CBP on the HTLV-I TRE and the HTLV-I TRE has a direct role in the stable incorporation of Tax into DNA bound complexes. A model describing these interactions is described in figure 1.12.

These studies have been extended by the use of truncated isoforms of CREB. Giebler *et al.*,(1997) has used a truncated form of CREB that contains only the 72-aa bZIP binding domain called CREB-BR. CREB-BR was able to support recruitment of CBP(KIX) *in vitro* and supported transactivation *in vivo* but only when Tax was present. Since CREB-BR lacks the ser-133 and activation domains, this result implies that DNA-bound non-phosphorylated CREB serves only as a scaffold for Tax in the recruitment of CBP to DNA. Furthermore, co-transfection of just the KIX domain of CBP inhibited Tax activation of HTLV-I dependent transcription. This suggests that the KIX domain acts as a competitive inhibitor of Tax transactivation by occupying the CBP binding site on Tax.

1.9 Scope of this Thesis

Clearly, chromatin compaction has an important role in the regulation of gene transcription by repressing basal transcription and by active involvement in the processes of inducible transcriptional regulation (section 1.4). This transcriptional regulation by chromatin involves a number of proteins that are involved in targeting specific areas for decompaction or remodelling. These mechanisms are not mutually exclusive but are interrelated in such a way that the cell can control the precise levels of transcription.

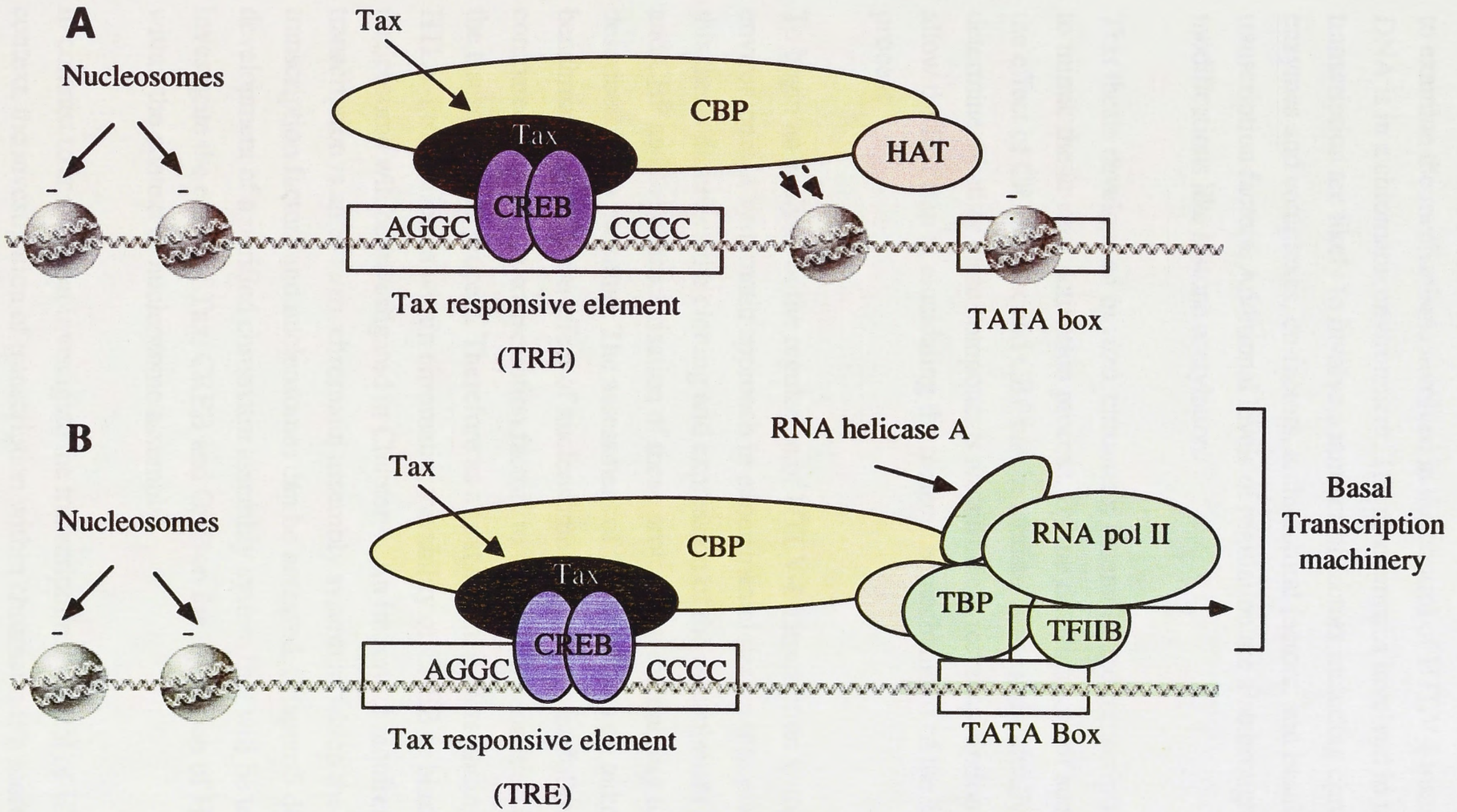


Figure 1.12: Model proposed for the activation of HTLV-I gene expression by Tax, CREB and CBP in a chromatin context. In this model CREB binds to the core CREB recognition sequence within the Tax responsive element (TRE). The virally expressed protein Tax, then binds to CREB recruiting the co-activator CBP. In the expression of cellular genes, CREB would normally require phosphorylation in response to the cellular signalling cascades in order to interact with CBP. The action of Tax recruiting CBP, therefore, bypasses the requirement for cellular signalling. Recruitment of CBP to the HTLV-I LTR may result in transcription activation by either **A.** Acetylation of core histone tails resulting in the destabilisation of nucleosome structure, or **B**. Direct interaction with the basal transcription machinery. The organisation of the HTLV-I nucleosomes has yet to be investigated and therefore the positions of the nucleosomes on the HTLV-I LTR are unknown. The nucleosomes in the figure are not positioned over particular sequence but represent the natural state of chromatin compaction found *in vivo*.

Although there have been many advances in the field of HTLV-I transcriptional regulation, little is known about the role of chromatin in HTLV-I transcription. The aim of this thesis is to examine the mechanisms involved in the control of HTLV-I transcription when the viral DNA is in a chromatin environment. The mechanisms involved in the regulation of HTLV-I transcription are likely to involve a number of factors including chromatin modifying enzymes and complexes, co-factors, architectural factors, and basal and sequence specific transcription factors. Additional levels of regulation are also brought about by protein modifications like histone acetylation.

This thesis develops an *in vitro* chromatin assembly and transcription system in an attempt to mimic the *in vivo* activation process. By the development of such an *in vitro* system, the effect of CREB, Tax and CBP can be studied either individually and in combination to determine whether these components are sufficient for transcriptional activation. This will allow the first step in elucidating the molecular mechanisms of the HTLV-I activation process.

To begin an analysis of the regulation of HTLV-I transcription within a chromatin environment, a systematic approach in experimental design was performed. Chapter 3 of this thesis describes the cloning and expression of the recombinant proteins Tax, CREB and CBP and the characterisation of these proteins by their ability to perform as previously described in the literature. The assessment of these proteins on naked DNA also serves as a benchmark to which the affect of nucleosome assembly in the following chapters can be compared. Binding of transcription factors to the nucleosome is an important first step in the transcriptional process. Therefore as a first step in understanding the regulation of HTLV-I transcription within chromatin, the ability of CREB to bind the HTLV-I nucleosome will be investigated in Chapter 4. An important requirement for the study of transcription is an *in vitro* chromatin assembly system in which the interactions between transcription factors and nucleosomes can be assessed. Chapter 5 describes the development of a purified chromatin assembly system that will be used in Chapter 6 to investigate the effect of Tax, CREB and CBP on the regulation of HTLV-I transcription within the context of nucleosome assembly.

In contrast to studies that investigate the transcriptional control of HTLV-I in a naked DNA context, the investigation of transcription within chromatin is a more extensive approach to determine the true molecular mechanisms which underlie the control of HTLV-I gene expression. By comparison with other studies that use artificial promoters and chimeric

transcription factors, this thesis investigates the interactions between full length natural proteins and their natural binding sites. Thus, this study represents a comprehensive investigation of the control of HTLV-I viral expression.

CHAPTER 2:

Materials and Methods

2.1 Recombinant DNA methods

2.1.1 Minipreparation of plasmid DNA

Small scale plasmid DNA (approx. 5 μg DNA) was prepared from an overnight bacterial culture in Luria broth (LB) [1% bactotryptone, 0.5% yeast extract, 1% NaCl (pH7.0)] supplemented with the required antibiotic (usually 100 μg/ml ampicillin) using the WIZARD Miniprep DNA purification system (Promega) according to the manufacturers instructions. Briefly, a 1-3ml overnight culture of *E.coli* was pelleted by centrifugation (14 000 x g). Plasmid DNA was released by alkali lysis of the cells followed by neutralisation and precipitation of the cellular debris. Plasmid DNA was purified by adsorption to the supplied resin and recovered by elution into either $T_{10}E_{0.1}$ pH 8 buffer of nuclease free MQ ddH_20.

2.1.2 Large scale preparation of plasmid DNA

Large scale DNA (approx 500 μg DNA) was prepared using the Qiagen Maxiprep purification system according to the manufacturers protocol. Briefly, a 100 ml LB culture (100 μg/ml ampicillin) was grown for 16 hrs at 37°C and 260 rpm. The cells were harvested by centrifugation at 6000 x g and lysed by alkaline lysis followed by binding of the DNA to a anion-exchange resin under low salt and pH conditions. RNA, proteins and low molecular weight impurities were removed by a medium salt wash. Plasmid DNA was eluted in a high salt buffer, concentrated and desalted by isopropanol precipitation

2.1.3 Quantitation of nucleic acids

Quantitation of DNA was performed by measuring the optical density (OD) at 260 nm and the concentration calculated using the relationship that at 260 nm, an OD of 1 is equivalent to 50 μg/ml of double stranded DNA (Sambrook *et al.*, 1989).

2.1.4 Restriction enzyme digestion of DNA

Restriction endonucleases were supplied by either Promega, New England Biochemicals (NEB) or Boehringer Mannheim. Digestion of DNA with restriction endonucleases was performed according to the manufacturers protocols and in the appropriate buffers specified for each enzyme.

2.1.5 Phenol/chloroform/isoamyl alcohol purification of DNA

To purify DNA for further down-stream applications an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) was added to the DNA solution and vortexed. After centrifugation at 14000 x g in a benchtop centrifuge, the upper aqueous layer was transferred to a new eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, vortexed and centrifuged before transferring the aqueous phase to a new tube. DNA was precipitated by the addition of 1/10 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol and incubated on dry ice for 20 min. The purified DNA was pelleted by centrifugation at 14 000 x g for 20 min, washed with 400 µl 70% (v/v) ethanol and vacuum dried for 10 min. The desiccated DNA was then resuspended in the appropriate buffer.

2.1.6 Blunt-ending DNA fragments

The 5'-protruding ends of DNA fragments created by restriction enzyme digestion were filled to generate blunt-ended DNA fragments by treatment with the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Typically, 1 U of enzyme per µg of DNA was used in a 20 µl reaction mixture containing 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 0.5 mM each dNTP. The reaction was incubated for 10 min at rt and terminated by incubation at 75 °C for 10 min.

2.1.7 De-phosphorylation of vector DNA

Following restriction digestion, phenol/chloroform/iosamyl alcohol extraction and ethanol precipitation, DNA was resuspended in reaction buffer (50 mM Tris-HCl pH 8.5, 0.1 mM

EDTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'- tetraacetic acid) pH 8.0. 2 U of Calf Intestine Alkaline Phosphatase (CIP) (Boehringer Mannheim) was added and the reaction was incubated at 37°C for 15 min. The reaction was terminated by the addition of 0.5 % SDS, 5 mM EDTA pH 8.0 and 100 μg/ml Proteinase K (Boehringer Mannheim) and incubated at 55°C for 30 min. The reaction mixture was purified by phenol/chloroform extraction and ethanol precipitation.

2.1.8 Agarose gel electrophoresis of DNA

Horizontal agarose gel electrophoresis was used to separate and purify DNA fragments generated by restriction enzyme digest of plasmid DNA. Electrophoresis was carried out using a voltage gradient of between 1-5 V/cm using 1 x TAE running buffer (40 mM Tris-acetate, 1mM EDTA pH 8.0). Prior to loading, the DNA samples were mixed with 1/10 volumes of 10 X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose).

2.1.9 Agarose gel purification of DNA fragments

To purify DNA fragments for cloning, the Bresaclean (Gene Works) system of DNA purification was employed according to the manufacturers instructions. Briefly, DNA restriction fragments were extracted from an agarose gel using a chaotropic salt (NaI) and adsorbed to a silica matrix. The matrix was washed to remove impurities and the DNA recovered by elution into TE.

2.1.10 Ligation of DNA termini

Typically a 5 molar excess of insert DNA to vector DNA was used in cohesive end ligation reactions. For blunt-end ligations a 10- to 20- fold molar excess of insert DNA was used. For both types of ligations, the reaction was carried out in a 10-20 μl volume containing 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 1-3 weiss units of T4 DNA ligase (Promega). The reactions were incubated at 16°C for 16-18 hrs before termination of the reaction by heating at 65°C for 15 min. Samples were diluted with an equal volume of H_2O for transformation.

2.1.11 Preparation of competent *E. coli* bacterial cells

A 5 ml LB culture was inoculated with a single colony from freshly streaked plates of the desired bacterial strain and incubated 16 hrs at 37°C, 260 rpm. This culture was used to inoculate a 500 ml LB culture which was grown at 37°C, 260 rpm until the absorbance at 600 nm was between 0.5 and 1.0. The culture was chilled on ice for 15-30 min before centrifugation at 6000 x g in a GS3 rotor at 4°C for 15 min. The bacterial pellets were resuspended in 500 ml of ice cold MQ ddH_2O and re-centrifuged. The cells were resuspended in a further 250 ml MQ ddH_2O and the centrifugation repeated. The pellet was resuspended in 10 ml cold 10% glycerol and centrifuged at 7000 x g for 10 min at 4°C. The cells were then resuspended to a final volume of 1.5 ml cold 10% glycerol (approx 1-3 x 10^{10} cells/ml). The competent cells were aliquoted into 45 µl amounts and stored at -70°C.

2.1.12 Transformation of electro-competent *E.coli*

Supercoiled plasmid DNA or 2 µl of diluted ligation reaction was mixed with 45 µl of electro-competent cells on ice. *E-coli* were transformed by electroporation using the Bio-Rad gene pulser II set at 2.5 KV and a capacitance of 25 µF with 200 ohms resistance. The transformed cells were immediately recovered in 1 ml of LB at 37°C for 1 hr before selection on LB agar plates containing the appropriate antibiotic (usually ampicillan 100 µg/ml).

2.1.13 DNA Sequencing

All plasmid constructs generated in this study were verified by sequence analysis. DNA sequencing was performed using the ABI prisim cycle sequencing system following the manufacturers instructions using appropriate primers. Electrophoresis of the sequencing reactions and automated sequence data collection were carried out by staff of the Biomolecular Resource facility (JCSMR).

2.2 General protein methods

2.2.1 Protein quantitation

The concentration of the recombinant proteins and protein fractions was determined using the BCA protein assay reagent kit (Progen) or the BioRad protein concentration reagent according to the manufactures instructions. Concentrations were determined by measuring colour change in a spectrophotometer and relating this value to a standard curve of known BSA concentration prepared in the same buffer.

2.2.2 SDS-PAGE

Discontinuous gels for SDS-PAGE were prepared and run according to the method of Sambrook *et al.*, (1989) using polyacrylamide:bis (29:1) (BioRad). Gels were prepared and run using a BioRad mini PROTEAN II gel apparatus. Before loading, the gel samples were mixed with an equal volume of SDS-PAGE loading buffer (30% sucrose, 5 % SDS, 1.5 M urea, 150 mM Tris-HCl pH 7.5, 0.3 mg/ml bromophenol blue, 3% (v/v) β-Me). Gels were visualised by staining with either Coomassie brilliant blue or silver according to established proceedures (Sambrook *et al.*, 1989).

2.2.3 Western analysis

Western analysis was performed according to Sambrook *et al.*, (1989). After separation by SDS-PAGE, proteins were transferred to nylon (PVDF) membranes (Novex) using the BioRad mini PROTEAN II transfer apparatus in transfer buffer (25 mM Tris-HCl pH 8.0, 1.92 mM glycine, 10 % methanol) at 100 V for 60 min. The membrane was washed with three changes of MQ ddH_2O before blocking overnight in PBST (PBS containing 5% tween-20) containing 5% non-fat milk powder. Membranes were washed in 3 changes of PBST and incubated for 1 hour with 1:4000 dilution of anti-Tax serum (kindly supplied by K. -T. Jeang, National institutes of Health, Besesthda, Maryland USA) or anti-CBP (Santa Cruz) in PBST. The membrane was washed in 3 changes of PBST before incubation for 1 hour with anti-rabbit IgG-Alkaline Phosphatase conjugate (Sigma) diluted 1:20000 in PBST. The membrane was washed in three changes of PBST and developed with instant developer (BioRad).

2.3 Protein purification

2.3.1 Expression and purification of recombinant transcription factors

2.3.1.1 Purification of CREB from *E-coli*

The Rat-$CREB_{341}$ cDNA expression clone (pET-CREB) was supplied in pET15b (Novagen) (without a 6 x His tag), a kind gift from R. H. Goodman (Vollum institute Oregon, USA).

The Initial purification strategy was based on the heat purification method of Loriaux *et al.*, (1993). pET-CREB was transformed into the BL21 (DE3) strain of *E.* (section 2.1.12) and grown to an OD_{600} of 0.6 in a 400 ml LB culture (100 µg/ml ampicillin) at 37°C and 260 rpm. Cells were induced to express recombinant CREB by the addition of 0.5 mM IPTG and the incubation was continued for a further 3 hrs. The cells were harvested by centrifugation in a GS3 rotor at 2500 rpm at 4°C for 10 min. The cells were resuspended in 16 ml of resuspension buffer [50 mM DTT, 50 mM EDTA, 10 µg/ml PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin, in phosphate buffered saline (PBS)]. Cells were lysed by sonication on ice at full power (Ystrom sonicator) for 40 seconds, 10 times, with 40 sec on ice between each sonication. Bacterial debris was cleared by centrifugation in a SS-34 rotor at 14000 rpm. The supernatant was divided into 16 1 ml aliquots in eppendorf tubes and heated at 72°C for 7 min. The tubes were transferred to a bench-top centrifuge and the heat precipitated proteins were pelleted at 14000 x g for 15 min at 4°C. The cleared supernatant was combined and stored at -20°C.

Recombinant CREB was further purified by column chromatography on cellulose phosphate P11 resin (Whatman). 4 g of P11 resin was prepared by washing with 50 ml 0.4 M NaOH followed by 50 ml 0.4 M HCl and 50 ml of EB buffer (100 mM NaCl, 1 mM EGTA, 20 mM HEPES (*N*-2 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.0, 5 mM KCl, 1.5 mM $MgCl_2$ 10% (v/v) glycerol, 0.5 mM DTT). The slurry was transferred to a 10 ml poly-prep chromatography column (Bio-Rad) and left to settle for up to 16 hrs so that the column contained 1.8 ml resin. The resin was washed with 10 volumes of EB

buffer with addition of proteinase inhibitors (10 μg/ml PMSF, 2μg/ml leupeptin, 2 μg/ml pepstatin). The CREB supernatant was loaded onto the column slowly by gravity flow to alow maximum adsorption to the resin. The column was washed with 10 volumes EB buffer containing 300 mM NaCl. CREB was eluted from the column using EB buffer containing 1 M NaCl and was collected in 600 μl fractions. Peak CREB containing fractions were identified by SDS-PAGE, pooled and dialysed against 60 mM buffer D (60 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA pH 8.0, 20 mM HEPES pH 7.9, 0.5 mM DTT, 10 μg/ml PMSF, 2μg/ml leupeptin, 2 μg/ml pepstatin) for 5 hrs at 4°C and stored in aliquots at -70°C.

a. *In vitro* phosphorylation of CREB

A modified method from Zhao and Giam (1992) was followed to phosphorylate CREB *in vitro*. 1.6-3.2 ng of phosphocellulose purified CREB was mixed with 5 units of protein kinase A catalytic subunit (Sigma) in 1x kinase buffer (50 mM KH_2PO_4/K_2HPO_4 pH7.2, 10 mM $MgCl_2$, 0.06 mM ATP) and incubated at 37°C for 15 minutes. Phosphorylation was monitored by the incorporation of [γ-^{32}P]dATP.

2.3.1.2 Purification of C-terminally tagged Tax (C-Tax) from *E-coli*

a. Protein expression

The C-terminally 6 x His bacterial expression construct Pet-Tax(H6) was a kind gift from C.Z. Giam (Uniformed Services University of Health Sciences Bethesda, Maryland USA). The strategy for the expression and pufication C-Tax was based on Tang *et al*. (1998). Pet-Tax(H6) was transformed into BL21 (DE3) cells and a 2 L culture was grown to an OD_{600} of 1.0-1.5 in selective LB medium (100 μg/ml ampicillin). Protein expression was induced by the addition of 40 μM IPTG at rt for 16 hrs. Cells were harvested by centrifugation in a GS3 rotor at 4000 rpm for 10 min at 4°C. The pellets were resuspended in 20 ml of NTB buffer [PBS (pH 8.0) containing 300 mM NaCl, 100 μg/ml PMSF, 0.5 mM β-mercaptoethanol, and 10 mM imidazole] and cells were lysed by sonication on ice at full

power (Ystrom sonicator) for 40 seconds, 10 times, with 40 sec on ice between each sonication. The resuspensions were cleared by centrifugation in a Sorvall SS-34 rotor at 14000 rpm for 50 min at 4°C.

b. Preparation of the ~~nickle~~ nickel-nitrilo-tri-acetic acid (Ni-NTA) column

2 ml Ni-NTA resin (Qiagen) was washed by adding 10 volumes of NTBbuffer and centrifuged for 3 min in a Beckman Allegra 6G clinical centrifuge at 1000 rpm. The supernatant was discarded and the washing step was repeated before resuspenion of the resin in an equal volume of NTB buffer.

c. Purification of C-Tax

The supernatant was mixed with 1ml of washed and equilibrated Ni-NTA resin. This mixture was incubated on a rotating platform at 4°C for 4 hrs and pelleted by centrifugation at 1000 rpm for 5 min at 4°C in a Beckman Allegra 6G clinical centrifuge. The pelleted resin was resuspended in 2 ml of NTB buffer and transferred a 10 ml poly-prep chromatography column (Bio-Rad). The column was washed with 10 volumes of NTB containing 40 mM imidazole before elution in 300 µl fractions with NTB buffer containing 100 mM imidazole. Peak Tax containing fractions were pooled and dialysed against buffer containing 20 mM HEPES pH7.9, 150 mM KCl, 0.2 mM EDTA, 10 µg/ml PMSF, 0.5 mM DTT, 20% (v/v) glycerol, 2 µg/ml leupeptin, 2µg/ml pepstatin) and stored in aliquots at -70°C.

2.3.1.3 Other bZIP proteins

ATF-2 was supplied by A. Henderson (JCSMR). c-Fos, c-Jun and AP-1 were supplied by M. Bunce and A. Henderson (JCSMR). All proteins were tagged with 6 x His and purified by nickel chromatography based on the method of Thanos and Maniatis (1996).

2.3.1.4 Purification of HMG-I from human placenta

HMG-I was prepared from human placenta by extraction with perchloric acid and purified by phosphocellulose and blue Sepharose chromatography as described in Lund *et al.*, 1987).

2.3.2 Expression and purification of transcription factors in the Baculovirus system

a. Tissue culture maintenance and propagation of SF-9 cells

The *Spodoptera frugiperda* (SF9) cell line was obtained from Dr. M.Summers (Jarvis and Summers, 1989). Typically, SF9 cells were cultured in 4 ml Sf-900 II serum free medium (SFM) (Gibco BRL) at 27°C with loose caps to allow gas exchange. Cells were grown to a confluent monolayer in 25-cm^2 flasks (Nunc) and once confluent, cells were resuspended by pipetting the medium across the monolayer with a pasture pipette. 2 x 10^5 viable cells/ml were then transferred to new flasks.

b. Construction of transfer plasmid from pFastbac encoding Tax and CPB cDNA

The entire cDNA for HTLV-I Tax was obtained from pGalTax, a kind gift from K. -T. Jeang (National institutes of Health, Besesthda, maryland USA). Tax cDNA was excised form pGalTax with *Eco*RI and *Xba*I and sub-cloned into the baculovirus transfer vector pFastBac Hta (digested with the same enzymes) in frame with the 6 x His tag according to section 2.1, to create pTax-Bac

The entire cDNA for mouse CREB Binding Protein (CBP) (approx. 8 Kb) was obtained from pRc/RSV/CBP, a generous gift from R.H. Goodman (Vollum Institute). CBP cDNA was excised form the plasmid using *Bam*HI (removing the HA tag and stop codon). This fragment was sub-cloned into pFastBac Htb linearised with *Bam*HI and dephosphorylated (according to section 2.1), in-frame with the 6 x His tag to create pCBP-Bac. Importantly pCBP-Bac was sequenced to check for the presence of a common mutation that results in a truncated CBP product (R.H Goodman, personal communication). Recombinant pFastpac

DNA was recovered from the Top10 F' cells used for sub-cloning using the wizard miniprep system (section 2.1.1) for transposition.

c. Generation of recombinant bacmids by transposition

To generate recombinant baculoviruses encoding CBP and Tax, the expression cassette from the pFastBac transfer vectors was transformed into *E.coli* DH10Bac (Gibco BRL) cells which contained the baculovirus shuttle vector (bacmid) (bMON14272) (Gibco BRL). Transposition of the pFastBac transfer vectors was carried out using a modified method from the Bac-to-Bac Baculovirus Expression Systems instruction manual (Gibco BRL). 1 μg of recombinant donor plasmid was mixed with 100 μl of DH10Bac competent cells and incubated for 30 min on ice. The mixture was heat shocked at 42 °C for 45 sec and immediately chilled on ice for 2 min. 900 μl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM ~~$Mg(SO)_4$~~ $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose) was added to the cells and incubated for 16 hours at 37°C with medium agitation. Cultures were centrifuged at low speed in a bench-top centrifuge, resuspended in 100 μl LB and plated onto selective LB plates (50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml X-gal, 40 μg/ml IPTG). Plates were incubated at 37°C for at least 24 hrs. White colonies were assayed for the presence of recombinant bacmid by PCR.

d. PCR analysis for recombinant bacmid

White colonies were transferred to 50 μl of MQ ddH_2O with a sterile toothpick and incubated at 65°C for 10 minutes to lyse the bacterial cells. Each colony was also touched to a numbered grid plate. 5 μl of lysate was amplified in a 20 μl reaction containing 1 x PCR buffer (Perkin Elmer, USA) (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 1.5 mM $MgCl_2$), 5 pmol of each primer (-40L and -44R, for Tax; Pbac2 and sGEX1 for CBP, table 2.1) 0.2 U of Taq polymerase (Amplitaq, Perkin Elmer) and 0.2 mM with respect to each dNTP. The PCR reaction was performed in capillaries in a Corbett Capillary Thermal Sequencer.

Primer	Sequence	Bacmid tested
-40L	5'-GGTTTTCCCAGTCACGACGTTG-3'	Tax
-44R	5'-GTGTGGAATTGTGAGCGGATAAC-3'	Tax
PBac2	5'-CATTCCTTTTATGTTTCAGGTTCAG-3'	CBP
sGEX1	5'-TGATGCAGCCTACTACAGCTA-3'	CBP

Table 4.2 PCR primers used for the verification of recombinant bacmids

Bacmid containing colonies were identified by the presence of a 4300 Kb band for Tax and 3800 Kb band for CBP. However, in each reaction, a band of 300 bp was also present indicating the presence of contaminating empty bacmid DNA in each of the colonies. Therefore, it was necessary to re-plate each of the colonies onto fresh selection plates and repeat the PCR analysis prior to isolation of bacmid DNA to ensure that there was no empty bacmid contamination.

e. Isolation of Bacmid DNA

Bacmid DNA was isolated according to the Bac-to-Bac Baculovirus Expression Systems instruction manual (Gibco BRL). Because the DNA to be isolated is very large, the preparation was carried out gently to avoid shearing the DNA. Selected colonies were used to inoculate a 2 ml LB culture (50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline) and incubated at 37°C, 260 rmp for 16 hrs. 1.5 ml cells was harvested by centrifugation at 14000 x g in a bench top centrifuge and resuspended in 300 μl of solution I (15 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 100 μg/ml RNase A). To lyse the cells 300 μl of solution II (0.2 M NaOH, 1% (v/v) SDS) was added to the resuspension and gently mixed before incubation for 5 min at rt. 300 μl of 3 M potassium acetate (pH 5.5) was slowly added and gently mixed during the addition. The preparation was centrifuged at 14 000 x g for 10 min at rt. The supernatant was gently transferred to a tube containing 800 μl of absolute isopropanol and gently mixed by inverting the tube several times before incubation on ice for 10 min. The preparation was centrifuged at 14000 x g for 15 min at rt to precipitate the DNA and the DNA was washed by replacing the supernatant with 70 % (v/v) ethanol. After centrifugation for 5 min, the 70% ethanol wash was repeated and the

pellet was air dried for 10 min. Finally the DNA was carefully resuspended in 40 µl TE pH 8.0 by gently tapping the tube over 10 min.

f. Transfection of recombinant baculoviruses into SF-9 cells

9 x 10^5 SF9 cells from a confluent monolayer culture (section a) were seeded in 35 mm wells of a 6 well plate in 2 ml of sf-900 II SFM and allowed to attach to the plate at 27°C for 1 hr. Prior to transfection, cells were washed twice with 2 ml of SFM. Each transfection was performed in duplicate. 5 µl of each bacmid DNA was added to 95 µl of serum free medium. 6 µl of CellFECTIN reagent (Gibco BRL) was added to 94 µl of SFM. The two solutions were combined and incubated at rt for 45 min. 800 µl of SFM was added to the DNA/CellFECTIN solution and the cells were overlayed with 1 ml of this solution, sealed with parafilm and incubated for 5 hr at 27 °C. The transfection solution was removed and replaced with 2 ml of SFM and incubated for 72 hrs. To harvest the virus, the media was aspirated from the cells and centrifuged for 5 min at 500 x g at rt. The virus containing media was transferred to a new tube and stored at 4 °C.

g. Viral amplification

To increase the viral concentration for protein production and purification, 2 x 10^6 cells in 2 ml was seeded into 6 well plates. 50 µl of virus from the original transfection was added to the media and incubated for 5 days before harvesting as above. The cells were washed with serum free media and lysed in 500 µl of SDS PAGE loading dye (section 2.2.2). Each sample was boiled for 3 min and 10 µl was assayed for protein expression by SDS-PAGE and western analysis (2.2.3).

h. viral plaque assay

In order to calculate the exact multiplicity of infection (MOI) of the viral stocks, viral plaque assays were performed. 8 serial log dilutions of amplified virus stock were created by sequentially adding 0.5 ml of the previous dilution to 4.5 ml SFM in 15 ml tubes. 2 x 10^6

cells were seeded into 6 well plates and allowed to adhere at 27 °C for 1 hr. The media was removed and the cells were overlayed with 1 ml of each dilution and incubated for 1 hr at 27 °C. The media was removed from high dilution to low dilution and replaced with 4 ml agarose media overlay (1.5 % low gelling temperature agarose (Edwards) 1 x Graces insect cell media (Gibco BRL). The agar was left to harden for 20 minutes before incubation at 27°C for up to 10 days.

i. Large scale protein expression and purification

50 ml spinner cultures were grown to 3 x 10^5 cell/ml and infected with 3.5 ml of amplified virus [approx 6.5 x 10^7 plaque forming units (PFU)/ml] for 72 hrs. The supernatant was cleared by centrifugation at 500 x g for 5 min and stored at 4 °C. This stock was used to infect large scale cultures for protein purification.

2 x 250 ml spinner flasks containing 2 x 10^6 cells/ml were infected with amplified virus (routinely 3 x 10^7 PFU/ml) at an MOI of 2.5 and incubated at 27°C for 72 hours. Cells were harvested by centrifugation at 500 x g at rt and the virus containing supernatant was stored at 4 °C as the viral stock for further protein purification preparations. The cell pellet was washed in SFM and resuspended in 2 volumes of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA. 0.5 mM PMSF, 1 mM DTT) and incubated on ice for 15 min. NP-40 was added to a final concentration of 1% (v/v) and mixed by pipetting with a 10 ml pipette and incubated a further 15 min on ice. The nuclei were pelleted by centrifugation at 500 x g for 10 min and the supernatant was carefully removed with a pasture pipette. The pellet was resuspended in 2 x the pellet volume of buffer C (20 mM HEPES pH 7.9, 420 mM NaCl, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), made up to 1% (v/v) NP-40 and gently stirred for 30 min at 4°C to extract soluble proteins. The preparation was transferred to eppendorf tubes and centrifuged at top speed in a bench top centrifuge for 15 min at 4°C. The supernatant containing soluble recombinant proteins was termed HSS1 and stored at -20°C. The pellet was resuspended in buffer C2 (840 mM NaCl, 20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) in a dounce homogeniser (loose fitting pestle). The resuspension was transferred to a small beaker and stirred for 30 min at 4 °C, centrifuged at 14000 x g in a

bench top centrifuge. The resulting preparation was termed HSS2 and stored at -20°C overnight.

Before affinity column purification, the HSS1 and HSS2 extracts were dialysed against loading buffer (50 mM Tris-HCl pH 8.5, 10 mM β-ME, 1 mM PMSF, 1% (v/v) NP-40, 10 μg/ml PMSF, 2 μg/m lpepstatin and 2 μg/m leupeptin) for 5 hr at 4 °C. After dialysis, the preparation was centrifuged at 14 000 x g in a bench-top centrifuge to remove any precipitated material.

A 1 ml nickel column was prepared by transferring 2 ml of Ni-NTA resin (Qaigen) equilibrated in buffer A (20 mM Tris-HCl pH 8.0, 500 mM KCl, 20 mM imidazole, 10 mM β-ME, 10% glycerol) to a 10 ml poly-prep chromatography column (Bio-Rad) at 4°C. The resin was left to settle and washed with 10 ml of buffer A at 4°C. The supernatant (either HSS1 or HSS2) was loaded onto the column and allowed to enter the column by gravity flow or pushed slowly through the column with a 5 ml syringe. The column was washed with 10 ml of buffer A followed by 2 ml of buffer B (20 mM Tris-HCl pH 8.5, 10 mM β-ME, 1M KCl, 10% glycerol, 10 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin). The recombinant proteins were eluted in 300 μl fractions with 10 ml of Buffer C (20 mM Tris-HCl pH 8.5, 100 mM KCl, 100 mM imidazole, 10 mM β-ME, 10% glycerol, 10 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin). 10 μl of each fraction was analysed by SDS-PAGE (section 2.2.2) and the peak protein containing fractions were dialysed for 5 hrs at 4°C against 60 mM buffer D (60 mM KCl, 20% v/v glycerol, 0.2 mM EDTA, 0.5 mM HEPES pH 7.9, 1 mM DTT, 10μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin), aliquoted and stored at -70°C.

2.4 Gel mobility shift and DNA footprinting assays

2.4.1 Construction and labelling of DNA probes

30 bp probes corresponding to various transcription factor binding sites on the HTLV-I and somatostatin regulatory regions were synthesised by the biomolecular resource facility

(JCSMR) and are outlined in table 2.2. The primers correspond to those regions indicated in figure 2.1. Complimentary primers were annealed by combining equimolar amounts of the primer pairs and incubating at 65 °C for 10 min. The primers were further heated at 100°C for 4 min before being left overnight at rt to anneal.

	Fragment	PRIMER 1	PRIMER 2
HTLV-I	DLTR	DLTR1 5'- AATTCAGACTAAGGCTC**TGAC** **GTC**TCCCCCCGGG-3'	DLTR2 5'- AATTCCCGGGGGGA**GACGT** **CA**GAGCCTTAG-3'
	MLTR	MLTR1 5'- AATTCAGGCCAGGCCC**TGACG** **TG**TCCCCCTGAAG-3'	MLTR2 5'- AATTCGGCAGGGGGA**CACGT** **CA**GGGCCTGGCCTG-3'
	PLTR	PLTR1 5'- AATTCCCTCAGGCGT**TGACGA** **C**AACCCCTCACCG-3'	PLTR2 5'- AATTCGGTGAGGGGTT**GTCG** **TCA**ACGCCTGAGGG-3'
Somato-statin	Somato-statin CRE	CRE1 5'- GATCGTCGACCTTGGC**TGACG** **TC**ACAGAGACGC-3'	CRE2 5'- GATCGCGTCTCTG**TGACGTC** AGCCAAGGTCGAC-3'
GAL4	GAL4	SOS5' 5'- GATCTGCTGTATATATATACAG CGCTACTGTATATACACCCAGG GC-3'	S0S3' 5'- GATCGCCCTGGGTCTATATAC AGTAGCGCTGTATATATATAC AGCA-3'

Table 2.2 DNA sequences of 30 bp TRE or CRE gel mobility probes. The TRE or CRE core elements are shown in bold. The nonspecific GAL4 probe used in competition experiments is also shown.

A variety of 147 to 180-bp DNA fragments were generated by PCR from the HTLV-I and somatostatin regulatory sequences employing the plasmids pHTBS and pSS-CRE (Goodman *et al.*, 1983) as the templates respectively (section 2.5.1). Specific primer pairs carrying various incorporated restriction enzyme sites (underlined) are outlined in table

Figure 2.1: Positions of primers on the HTLV-I and somatostatin sequences. The positions of the primers used to generate probes by PCR are indicated by arrows over or above the sequence. Sequences of the oligonucleotides used as the 30 bp probes are shown as a grey box. The core Tax responsive elements (TREs) (panel A) or the cAMP responsive element (CRE) (panel B) is shown as the central box. Sequence is numbered relative to the start site of transcription of HTLV-I (Panel A) or somatostatin (panel B). **A.** The template used for PCR was pHTBS (section 2.5.1) and the probes generated by PCR* were H147(M): -166 to -25; H147(E): -211 to -72; H180(2): -211 to -41. The 30 bp oligonucleotides spanned -109 to -82 (pLTR); -110 to -80 (mLTR); -231 to -261 (dLTR). **B.** The somatostatin template was pSS-CRE (section 2.5.1) and consists of the somatostatin promoter (-71 to +58) (dark type), CAT sequence (light type) 3' of the somatostatin promoter and pUC12 sequence (light type) 5' of the somatostatin sequence. The probe generated by PCR* was S180: -133 to +43. The 30 bp oligonucleotide CRE probe spanned region -57 to -27. (*The probes generated by PCR contained restriction enzyme sites at the 5' end and three further 5' nucleotides to ensure cutting, this extra sequence makes the length of the PCR product 147 or 180 bp).

2.3). Typically 1 ml PCR reactions containing 1μg of plasmid template, 250 pmol each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$ and 10 units of AmpliTaq polymerase (Perkin Elmer) in 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.001% (w/v) gelatin 1.5 mM $MgCl_2$). The PCR was carried out in 100 μl aliquots in a Perkin Elmer GeneAmp PCR system 2400 machine. PCR was denatured at 94°C for 5 min before 30 cycles at 94°C (15 sec), 58 °C (15 sec), 72°C (20 sec) followed by a final 10 min 72°C chase step. Following synthesis, DNA products were extracted with phenol-chloroform-isoamyl-alcohol, followed by ethanol precipitation. The DNA pellet, after drying in a Speed-vac, was resuspended in TE pH 8.0 and passed over a nick-spin column (Pharmacia) to remove unincorporated nucleotides. The amplified DNA products were quantitated using gel electrophoresis by comparison with known mass ladders (Gibco BRL).

	Fragment	primer	primer
HTLV-I	H147(E)	GSHT15' 5'-GTGGAATTCTCAGGCTAGGCCCTGACGT-3'	GSHT23' 5'GTGGGATCCTTTTTTGAGGTGAGGGGTTGT-3'
	H180(2)	GSHT15' 5'-GTGGAATTCTCAGGCTAGGCCCTGACGT-3'	GSHT43' 5'-GTGGGATCCGTTAGTTTATTCAGCCATATG-3'
	H180(3)	GSHT55' 5'-GTGGAATTCAAGGCTCTGACGTCTCCCC-3'	GSHT63' 5'-GTGGGATCCTGAGGGGTTGTCGTCAACGC-3'
	H147(M)	GSHT75' 5'-GTGGAATTCAGACCTCCGGGAAGCCACC-3'	GSHT83' 5'-GTGGGATCCTATAGACTCCTGTTAGTTTATTCA-3'
Somatostatin	S180	GSSO15' 5'-GTGGGATCCCAGGCAGCAGAGACGACTC-3'	GSSO23' 5'-GTGGAATTCCCAGTCACGACGTTGTAAAAC-3'

Table 2.3: PCR primers used for the generation of 147-180 bp probes used for gel mobility shift reactions and as the template for single nucleosome reconstitution.

2.4.2 Radiolabelling of the 180 or 147 bp DNA fragments

For mobility shift and footprinting assays the various DNA fragments were digested with restriction enzymes in appropriate digestion buffers to create a 5' overhang. Typically 2.5 μg of the DNA fragment was digested for 2 hours at 37°C. Following digestion, the DNA was extracted with phenol/chloroform/isoamyl alcohol, ethanol precipitated and resuspended in 20 μl TE pH 8.0. The concentration of the DNA was determined by comparison with known mass markers on agarose gel electrophoresis.

The digested fragments were end labelled by Klenow incorporation of [α-^{32}P]dATP. In a reaction containing 500 ng DNA fragment, 1 x Klenow buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT), 0.5 mM of each dNTP (except ATP), 20 μCi of [α-^{32}P]dATP (3000 Ci/mmol, GeneWorks Australia) and 5 U Klenow polymerase (Bohringer Manhiem). The reaction was incubated for 30 min at 37°C with the addition of unlabelled nucleotides for an additional 5 min chase. The labelled fragments were separated from unincorporated nucleotides using Nick-Spin columns (Pharmacia) and further purified on an 8% acrylamide (acrylamide-bisacrylamide, 29:1)-1 X Tris-borate-EDTA (TBE) gel. The gel was autoradiograhed for 1 min and the fragments were excised from the gel and eluted in elution solution (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0 and 0.1% SDS) overnight at 37°C with rotary shaking. The radiolabelled fragments were ethanol precipitated, washed with 70% ethanol and dried under vacuum followed by resuspension in 12 μl TE pH 8.0. The specific activity of 1 μl of the DNA was assessed using a Liquid Scintillation Analyser (Packard, 1900CA) and diluted to 300 000 cpm/μl.

2.4.3 Binding reactions

For gel mobility shift reactions and footprinting using recombinant proteins, standard binding reactions were carried out in a final volume of 25 μl containing 56 mM KCl, 1 mg/ml BSA, 10 mM DTT and 0.5 μg poly (dG.dC) (Pharmacia). The concentrations of the recombinant proteins are sown in the figure legends. Either 15000 cpm (1.25 ng) of appropriate naked DNA or reconstituted nucleosome [assembled from chicken long

chromatin or acetylated HeLa chromatin (section 2.11)] probe was added to the binding reaction. Where N1/N2(H3,H4) wasbound to the probes, the binding conditions were 60 mM KCl, 26 mM EDTA, in 1 x EB buffer (1 mM EGTA, 20 mM HEPES pH 7.0, 5 mM KCl, 10 % (v/v) glycerol, 0.5 mM DTT, 10 mM β-glycerol phosphate). All binding reactions were conducted for 30 min at rt.

For mobility shift assays, binding reactions received 3 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose). Samples were loaded directly onto a pre-electrophoresed 4.5% non-denaturing polyacrylamide (acrylamide:bisacrylamide, 29:1)-0.5 x TBE gel and run at 15 V/cm at 4°C. Gels were dried and exposed to X-ray film (Kodak) at rt overnight.

For DNase I footprinting, after the 30 minute binding reaction, 2.5 µl of a 10 mM $MgCl_2$/5 mM $CaCl_2$ solution was added to the reaction. Binding reactions were digested with between 0.1 and 0.2 U of DNAse I (Boehringer-Mannheim) for naked DNA and between 0.8 and 1.2 units for protein bound DNA, for 1 minute at rt. Digestion was terminated by the addition of 180 µl of DNase I stop solution (100 mM NaCl, 15 mM EDTA, 0.5% (v/v) SDS and 50 µg/ml tRNA [Sigma]). The samples were extracted with 200 µl of phenol/chloroform/isoamyl alcohol and precipitated with 100% ethanol, washed with 70% ethanol and dried under vacuum. The pellets were resuspended in 3 µl of formamide loading dye (deionised formamide ([Fluka], 10 mM EDTA, 0.3% bromophenol blue and 0.3% xylene cyanol), heated for 5 min at 80 °C, placed immediately into an ice bath and loaded onto a pre-run 8% acrylamide/7M urea polyacrylamide gel. A G+A sequencing ladder specific for each probe under investigation was also loaded onto the gel (section 2.4.4). The samples were resolved at 1500 V for 2 hours before transferring the gel to DEAE ion-exchange paper (Whatman). The gel was dried under vacuum and exposed to X-ray film (Kodak) at -70°C.

2.4.4 G+A sequence markers

A G+A sequence marker was prepared for each probe under investigation. 2 x 10^5 cpm of labelled fragment was combined with 0.5 µg of poly (dG.dC) in 10 µl. 1 µl of 4% formic acid was added and the reaction was incubated at 37°C for 25 min. The samples were placed on ice and 150 µl of 1.0 M piperidine was added. Following heating at 90°C for 30 min, the reactions were incubated on ice for 5 min before the addition of 1 ml of butanol. The samples were vortexed, centrifuged at 16 000 x g for two minutes and washed twice in 500 µl butanol before drying under vacuum. The pellets were resuspended in 20 µl of formamide loading dye and stored at -20°C for up to 1 week.

2.5 *In vitro* transcription

2.5.1 Construction of templates

The template used for HTLV-I *in vitro* transcription comprised a 700 bp region of the 3' HTLV-I U3-R region from pU3RI-CAT (Cullen, 1986) (Kindkt supplied by Dr P. Morrow, Gladstone Institute of Virology and Immunology, California, USA). This 700 bp region was excised with *Hind*III and *Xho*I and sub-cloned into pBluescript II KS+ (Stratagene) which had been restriction digested with the same enzymes. The size of the resulting plasmid, HTBS was 3.4 Kb.

The template used for control *in vitro* transcription experiments was pSS-CRE supplied by R. H. Goodman. PSS-CRE contains approximately 150 bp of the somatostatin promoter and the CAT reporter gene within a pUC 12 plasmid vector and is 2.7 kb.

2.5.2 Primers

The primer HTCV-I was used for the primer extension from HTLV-I RNA templates and was designed from +45 to +70 on the HTLV-I LTR to yield a 70 bp product .

5'-AACGCGACTCAACCGGCGTGGATGGC-3'

The primer SOCV-I was used for somatostatin primer extension was designed from the p SynCat gene (SynapSys Corp., Burlington, MA, USA) and lies 95 bp upstream from the somatostatin transcription start site in pSS-CRE.

5'-TCCTGAAAATCTCGCCAAGCTT-3'

2.5.3 Primer labelling

1 pmol of primer was labelled with 20 μCi of [γ-^{32}P] ATP (3000 Ci/mmol: Geneworks) in a 10 μl reaction with 0.1 U Polynucleotide Kinase (PNK) (USB) in 1 x 1-phor-all buffer (Pharmacia). The reaction was incubated at 37 °C for 30 min, heat inactivated at 90°C for 2 min and diluted to 10 fmol/μl with TE buffer pH 8.0.

2.5.4 Nuclear transcription extract preparation

Nuclear extracts were prepared from the Jurkat T cell line by a method modified from Dignam *et al.* (1983). All procedures were performed at 4°C. Typically 20 to 40 200 ml Jurkat cell cultures were grown to 2 x 10^7 cells/ml (Section 2.12) and harvested by centrifugation for 10 min at 2500 rpm in a Sorvall GS3 rotor. The cells were gently resuspended in 20 ml PBS and combined into two 500 ml GSA buckets and re-centrifuged. The supernatant was removed and the cells were resuspended in 30 ml PBS and transferred to one SS-34 rotor tube and centrifuged at 2500 rpm for 10 min. The supernatant was removed and the pellet was resuspended in 5 volumes of buffer A (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES pH 7.9, 0.5 mM DTT, 0.5 M PMSF, 2 μg/ml pepstatin and 2 μg/ml leupeptin) and centrifuged at 2500 rpm for 10 min. The pellet was resuspended in 2 volumes of buffer A and lysed in a dounce homogeniser using 10 strokes of the tight fitting pestle. After checking for cell lysis under a microscope, the released nuclei were pelleted by centrifugation at 2500 rpm for 10 min and the supernatant was decanted carefully using a pasture pipette. The pellet was re-centrifuged at 25 000 x g for a further 10 min and the supernatant was removed. The pellet was resuspended in 2 volumes of buffer C (1.1 mM $MgCl_2$, 0.42 mM NaCl, 26% glycerol, 0.15 mM EDTA, 15 mM HEPES pH 7.9, 0.5 mM DTT, 0.5 M PMSF, 2 μg/ml pepstatin and 2 μg/ml leupeptin) using 10 strokes of the

dounce homogeniser, transferred to a glass beaker and stirred gently for 30 min. The extract was transferred to SS-34 rotor tubes and centrifuged at 25 000 x g for 30 min. The supernatant was made up to 8 ml with buffer C, transferred to another beaker and 0.36 g/ml solid ~~$NH_4(SO)_2$~~ $(NH_4)_2SO_4$ was added slowly over 5 minutes with stirring. Once dissolved, 1 µl/ml of 1 M NaOH was added to the extract and stirred gently for 30 min. The precipitate was collected by a 15 minute centrifugation step at 25 000 x g and resuspended in 0.5 x the original volume (before being made up to 8 mls). The extract was dialysed against buffer D (100 mM KCl, 20 % (v/v) glycerol, 0.2 mM EDTA, 20 mM HEPES pH 7.9, 0.5 mM DTT, 0.5 M PMSF, 2 µg/ml pepstatin and 2 µg/ml leupeptin) for 5 hours. After dialysis, the extract was centrifuged at top speed in an eppendorf centrifuge and stored in liquid nitrogen in 100 µl aliquots. The concentration of the extract was determined using the Bio-Rad protein assay according to the manufactures instructions and was typically 2-5 mg/ml.

2.5.5 Transcription and primer extension

In vitro transcriptions were performed with typically 300 ng of DNA template in a 60 µl volume consisting of 5 mM $MgCl_2$, 65 mM ~~salt~~ NaCl, 65 U RNasin (HPR I) (Amersham) and 75 µg nuclear extract (section 2.5.4). Reactions were incubated at 30°C for 30 min to allow for the formation of transcriptional pre-initiation complexes before the addition of 0.625 mM each rNTP to start the synthesis of RNA. Where template DNA was assembled into nucleosomes by the NAP-1 system (section 2.11.2.2), or S-150 system (section 2.11.2.4), ATP was omitted as ATP was already added in the assembly reaction. These conditions were determined empirically and may vary for some experiments as indicated.

RNA systhesis was carried out at 30°C for 30 min before the addition of 2 x stop buffer (0.8% SDS, 40 mM Tris-HCl pH 7.5, 16 mM EDTA, 80 µg/ml tRNA, 320 µg/ml proteinase K). The reaction was further incubated for 60 min at 40°C. The RNA was extracted with an equal volume of phenol /chloroform/isoamyl alcohol (25:24:1) (Sigma). To maximise recovery, the phenol was back-extracted with 50 µl of T.E buffer (pH 8.0) before ethanol precipitation. Precipitated DNA was washed with 70% ethanol and dried under vacuum.

For primer extension, the RNA pellet was resuspended in 6 µl TE buffer (pH 8.0) and after the addition of 2 µl of annealing buffer (10 mM Tris-HCl pH 7.9, 1 mM EDTA, 1.25 M KCl) and 2 µl of labelled primer (20 fmol) (section 2.5.3) the reaction was denatured at 75°C for 30 min. After denaturation, the primer was allowed to anneal to the RNA template by slow cooling to 37°C over 30 min. 12.5 µl of priming buffer (40 mM Tris-HCl pH 8.7, 20 mM $MgCl_2$, 10 mM DTT), 65 U RNAsin and 1 mM final concentration of each dNTP was added to the reaction and incubated at 42°C for 5 min. The reverse transcriptase (30 U/µl, AMV) was diluted to 3 U/µl in 1 X buffer (50 mM Tris-HCl, pH 8.3, 40 mM $MgCl_2$, 250 mM NaCl, 5 mM DTT) and 2 µl (6 U) was added to the reaction and incubated for 45 min at 42°C. The DNA was recovered by ethanol precipitation, resuspended in 4 µl of formamide loading dye (5 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue in de-ionised formamide) and separated on an 8 % acrylamide sequencing gel (acrylamise:bis, 19:1/ 7 M urea/1 X TBE) at 1500 volts. The gel was transferred to DEAE ion-exchange paper (Whatman), dried under vacuum and exposed to either X-ray film (Kodak) or a phosphoimage screen (FujiFilm). Transcription products were quantitated using a FLA-300 phosphoimager and image gauge (Fuji Film) software.

2.6 Exonuclease III assay

2.6.1 Probe labelling

In order to label the 180 bp somatostatin probe (S180) on the 5' end of one strand, the 180 bp PCR product (section 2.4.1) was digested with *Eco*R1 (or *Bam*HI for the opposite strand) as in section 2.4.2, extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated. 2.5 µl (approx. 500 ng) digested fragment was then labelled in a 10 µl reaction with 1.0 U PNK (USB) in 1 X 1-phor-all buffer as in section 2.2.5.3 and purified on a Nick Spin column. To remove the 5' label from one strand, the probe was then digested with BamH1 (or *Eco*R1 for the opposite strand) and gel purified according to section 2.4.2). The probes were quantitated by counting as described in section 2.4.2.

2.6.2 Exonuclease III digestion

Probes were assembled into single nucleosomes as described in section 2.11.2.1 and incubated at 37°C for 1 hour. CREB was bound to the nucleosome in a standard binding reaction according to section 2.4.3. After electrophoresis on a non-denaturing 4.5% polyacrylamide gel, the nucleosome bands were excised and transferred to tubes containing 500 µl of equilibration buffer (10 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 5 mM β-ME). The gel slices were incubated at 24°C for 15 minutes. Exonuclease III (Pharmacia) was diluted to 7.5 U/µl in equilibration buffer. The gel fragments containing the nucleosome bands were digested with either 15, 50 or 150 units of Exonuclease III at 24°C for 30 min. The gel slices were transferred to 500 µl of elution solution (section 2.4.2) and digested with proteinase K (100 µg/ml) at 37°C overnight. The gel slices were removed from the solution and the samples were ethanol precipitated, washed with 70% ethanol, resuspended in 4 µl of formamide loading dye and run on a 7 M urea, 8% acrylamide (acrylamide-Bis 19:1), 1 X TBE sequencing gel.

2.7 Cross-linking

2.7.1 Formaldahyde cross-linking

The method for the creation of DNA-protein cross-links was based on Jackson (1999). 25 ng of end labelled DNA fragment (32 to 3.5 x 10^5 cpm) was assembled into a single nucleosome according to section 2.11.2.1. After dilution to 100 mM NaCl, formaldehyde was added to a final concentration of 1 % and incubated at 0°C for 18 hours. The formaldehyde was removed by dialysis against final dilution buffer (10 mM HEPES, 1mM EDTA, 0.1% (v/v) NP-40, 20 % (v/v) glycerol, 5 mM DTT, 0.5 M PMSF, 2 µg/ml pepstatin and 2 µg/ml leupeptin) at 4°C overnight. Reactions were resolved on 18% SDS-PAGE (section 2.2.2) to determine the completeness of the cross-linking reaction.

2.7.2 Dimethyl suberimidate cross-linking

The method used for the creation of protein-protein cross-linking with dimethyl suberimidate was based on the methods of Walter *et al.* (1995). Approximately 25 ng of end labelled DNA fragment (3 to 3.5 x 10^5 cpm) (section 2.4.3) was mixed with H1 depleted oligonucleosomes from chicken erythrocytes (section 2.11.1.1) at 1 M NaCl in a 20 µl reaction volume. Following incubation at 37°C for 30 minutes, the reaction was diluted (3 steps) to 0.4 M NaCl in a 50 µl final volume with 10 mM HEPES pH 7.5, 1mM EDTA with a 25 minute at 30°C between each dilution step. The reaction was then further diluted to 0.1 M NaCl (3 steps) with 100 mM sodium borate, pH 10.0. Dimethyl suberimidate (prepared as an 11 mg/ml stock solution in 100 mM sodium borate, pH 11) was added to a final concentration of 2 mg/ml and incubated for 45 minutes at 45°C. The dimethyl suberimidate was removed from the reaction by dialysis against 25 mM Tris-HCl, pH 6.8 and 1 mM EDTA, pH 8.0 for 90 min at 4°C. The reaction was dialysed in final dilution buffer (10 mM HEPES, 1mM EDTA, 0.1% (v/v) NP-40, 20 % (v/v) glycerol and 5 mM DTT , 0.5 M PMSF, 2 µg/ml pepstatin and 2 µg/ml leupeptin) overnight at 4°C. Control reactions were processed in the same way but contained sodium borate in place of dimethyl suberimidate. Reactions were visualised by 18% SDS-PAGE to determine the completeness of the cross-linking reaction.

2.8 Supercoiling assay

300 ng of plasmid template DNA was combined with 2 units of topoisomerase I (Promega, 10 U/µl) and incubated at 30°C for 1 hr to relax the DNA. The components of either the S-150, N1/N2(H3,H3) or NAP-1 assembly systems were combined with the relaxed DNA template and incubated at 30°C or 27°C for 4 hrs as described (section 2.11.2). Assembly reactions were brought to a volume of 50 µl before the addition of 12.5 µl of sarkosyl EDTA solution (100 mM EDTA, 12.5% v/v sarkosyl), 8 µl of 2% SDS and 8 µl of proteinase K (10 mg/ml). The reaction was incubated at 37°C overnight before precipitation with 53 µl 7.5 M ~~$(NH_4)_2OAc$~~ $NH_4C_2H_3O_2$ and 265 µl ethanol. After washing, in 70% ethanol, the pellet

was resuspended in 10 µl of 1 X loading solution and run on a 1% agarose gel at 100 volts for 4-5 hours (section 2.1.9). DNA was visualised by ethidium bromide staining.

2.9 Micrococcal nuclease assay

Assembly reactions were prepared as described in section 2.11.2 and incubated for 4 hours at 37°C or 27°C. After the addition of $CaCl_2$ to a final concentration of 3 mM, 50 units of micrococcal nuclease S7 (Roche) was added and the reaction was incubated at rt for the indicated time period. After each time period the reaction (100 µl) was transferred to a fresh tube containing 25 µl 2.5% sarkosyl and 10 mM EDTA. 1U heat inactivated RNase A (Sigma) was added to the reaction and incubated for a further 60 min 37°C. 16 µl of 2% SDS and 16 µl of proteinase K (10 mg/ml) was added and the reaction was incubated at 37°C overnight. The reactions were precipitated with 106 µl $(NH_4)_2$oAc and 530 µl ethanol. The pellets were washed in 70 % ethanol and resuspended in 9 µl of sucrose/ EDTA solution (7% sucrose, 5 mM EDTA) and run on a 1.5% agarose gel at 100 volts for 5 hours.

2.10 Histone acetyltransferase assay (HAT assay)

Various proteins were acetylated *in vitro* based on the methods of Ogryzko *et al.* (1996) and Bannister and Kouzarides (1996). Reactions were performed in 1 X HAT buffer (50 mM Tris-HCl pH 8.0, 10 % (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM EDTA, 10 mM sodium butyrate pH 7.0). Protein substrate (recombinant proteins or purified histones) were mixed with CBP and 0.25 µCi (1 µl) of [^{3}H]Acetyl-coenzyme A (Amersham, 5.45Ci/mmol). Reactions were incubated at 30 °C for 1 hr and terminated by the addition of SDS-PAGE loading dye (section 2.2.2). Reactions were run on 10-18% SDS-PAGE, stained with Coomassie blue and destained (Section 2.2.2). Gels were soaked in Amplify (Amersham) for 30 min and dried under vacuum before exposing to pre-flashed film (Kodak) for up to 4 days.

Where proteins were acetylated for down-stream applications such as the gel mobility shift assay, un-labelled acetyl-coA (Sigma) was employed. 10-fold molar excess of un-labelled

acetyl-coA (in comparison with molarity of the protein to be acetylated) was used in place of [^{3}H]Acetyl-coenzyme A as described above.

Purified AP-1, c-Fos and c-Jun was supplied by M. Bunce and A. Henderson (JCSMR) (Henderson *et al.*, 2000). Purified PC4 was supplied by Dr. A. Holloway (JCSMR) (Holloway *et al.*, 2000). Purified histones H1, HMG-1 and HMG-14 were supplied by Dr. D. Tremethick (JCSMR) (Tremethick, 1994).

2.11 Chromatin assembly systems

2.11.1 Preparation of protein fractions

2.11.1.1 Preparation of long chicken donor chromatin

Chicken long chromatin stripped of linker histones and non-histone proteins, was prepared from chickens blood (Steggles Vaccine Laboratory) essentially according to the method of Drew and Calladine (1987). 20 ml of chicken blood was diluted to 160 ml with Buffer A [15 mM sodium cacodylate, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine 0.15 mM spermine pH 6.0] containing 0.34 M sucrose, 2.0 mM EDTA, 0.5 M EGTA, 0.5 M β-ME, 10 μg/ml PMSF and 1.0 mM benzamidine. The suspension was transferred to GS3 tubes and centrifuged at 2000 rpm for 3 minutes at 4°C. The cell pellet was washed in the above buffer 3 times before resuspension in 40 ml of the same buffer adjusted to 0.1% (v/v) NP-40 and pH 7.5 with Tris-base. The suspension was then centrifuged at 3000 rpm in a GS3 rotor and washed in the detergent solution until the pellet became milky-white in colour. The released nuclei were resuspended in 40 ml Buffer A containing 0.34 M sucrose, 10 μg/ml PMSF and 15 mM β-ME and centrifuged at 3000 rpm for 3 min. The pellet was resuspended in 6 ml of the same buffer and the concentration of DNA was determined from the absorbance at 260 nm. The absorbance was adjusted to 50 absorbance units /ml of nuclei (approx. 5 mg/ml).

For micrococcal nuclease digestion, the solution was adjusted to 1.0 mM $CaCl_2$ and incubated at 37°C for 3 minutes. Micrococcal nuclease (Sigma) was added to a final concentration of 0.5 U/ml and the incubation continued for a further 5 minutes. The digestion was terminated by addition of EDTA to 2 mM before centrifugation of the nuclei

at 5000 rpm. To lyse the nuclei, the pellet was resuspended in 10 ml (approx. 0.5 X previous volume) of 10 mM Tris-HCl pH 8, 0.2 mM EDTA, 10 µg/ml PMSF and incubated on ice for 30 min. In order to keep the chromatin in solution, the pH of the resuspension was closely monitored to ensure it remained above 7.5 and periodically shaken. After lysis of the nuclei, the solution was centrifuged at 5000 rpm for 10 min. The supernatant was measured and 4 M stock NaCl was added very slowly, drop-wise, until the chromatin solution contained 0.65 M NaCl, ensuring quantitative release of linker and non-histone proteins.

This preparation was applied to a Sepharose CL-4B column (2 x 20 cm) equilibrated with 0.63 M NaCl, 20 mM sodium cacodylate, 0.2 mM EDTA and 10 µg/ml PMSF (pH 6.0). The collected fractions were assayed for protein concentration by measuring the absorbance at 260 nm. Peak fractions were subjected to 15% SDS-PAGE in order to select fractions free from linker histones and other contaminating proteins. Peak, uncontaminated fractions were dialysed against two changes of 10 mM sodium cacodylate, 0.2 mM EDTA and 10 µg/ml PMSF (pH 6.0). The preparation was concentrated to 10 mg/ml using a centricon-30 concentrating cell (Amicon), diluted to 5 mg/ml with glycerol and stored at -20°C.

2.11.1.2 Preparation of S-150 assembly extract from *Xenopus laevis* oocytes

Oocyte extracts were prepared from adult *Xenopus laevis* female toads (Nasco) essentially as described by Gilkin *et al.* (1984) and Shimamura *et al* (1998). Ovaries were washed in OR-2 medium [5 mM HEPES pH 7.5, 1 mM Na_2HPO_4, 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$] before digestion for 3 hours with 0.15% (w/v) collagenase type II (Sigma) in OR-2 medium at rt. The dispersed oocytes were washed 10 times in OR-2 medium followed by three washes in low-ionic-strength extraction buffer (20 mM HEPES pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% (v/v) glycerol, 10 mM β-glycerophosphate, 0.5 mM DTT). Beckman SW41 centrifuge tubes were completely filled with oocytes and centrifuged at 40 000 rpm (150 000 x g) for 2 hours at 4°C in a Beckman L8-70 Ultracentrifuge. The clear supernatant, formed between the top fat layer and the yolk/insoluble debris at the bottom, was carefully removed with a syringe through the side

of the centifuge tube. Proteanase inhibitors (10 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin) were added and gently mixed with the supernatant.

2.11.1.3 Preparation of N1/N2(H3,H4) complex from *Xenopus laevis* oocytes

The S-150 oocyte extract prepared above was adjusted with 5M NaCl to a final conductivity of 0.1 M NaCl in buffer A (20 mM HEPES pH7.5, 0.1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μg/ml PMSF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) and loaded on a 1 ml column of DEAE-Sephacel (Pharmacia) equilibrated in the same buffer. The loading of the supernatant was carried out slowly at 4°C . After washing the column with 5 volumes of 0.1 M NaCl in buffer A (with fresh addition of protease inhibitors), The N1/N2-(H3,H4) complexes were eluted with 5 ml of 0.2 M NaCl in buffer A and collected in 300 μl fractions in 1.5 ml eppendorf tubes.

The fractions were assayed for protein concentration by mixing 10 μl from each fraction with 200 μl aliquots of protein assay dye reagent (Bio-Rad), diluted 1:5 with water. Fractions with the three highest concentrations were pooled and directly loaded onto two linear 5-20% sucrose gradients in buffer A containing 23 mM KCl, 17 mM NaCl, 20 mM HEPES pH 7.5, 0.1 mM EDTA, 2 mM $MgCl_2$, 1mM DTT, 10 μg/ml PMSF, 2 μg/ml leupeptin, and 2 μg/ml pepstatin. The two 12 ml gradients were centrifuged in a SW41 rotor at 150 000 x g for 24 hours at 4°C. Gradients were fractionated into approximately 24 fractions of 500 μl each, using a peristaltic pump (LKB Bromma Model 2120)/fraction collector (Gilson Microcol TDC 80) unit system. The three fractions centred at the 5S position on the gradient, which contained the highest levels of DNA supercoiling activity, were pooled and stored in 100 μl aliquots at -70°C until used (Worcel *et al.*, 1978).

2.11.1.4 Preparation of acetylated HeLa donor chromatin

HeLa cells were grown in suspension with Dulbecco's minimal essential medium supplemented with 10% FCS (v/v). Media containing 8 mM sodium butyrate (freshly

prepared stock) were supplied and the cells were grown for 24 hrs at 37°C. Cells were harvested by centrifugation 4°C for 10 min at 1000 x g and treated to prepare nuclei following the procedure of Ausio and van Holde (1986). Briefly, cell pellets were resuspended in buffer A (10 mM morpholineethane-sulphonic acid (MES), 0.25 M sucrose, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 10 mM sodium butyrate, 0.5% triton X-100. 0.1 mM PMFS) and centrifuged at 3000 x g for 10 min at 4°C. The pellet was resuspended and centrifuged twice more to yield purified HeLa nuclei. Pelleted nuclei were resuspended to a A_{260} of 40 in buffer B [10 mM PIPES {piperizine-N,N'bis[2ethanesulphonic acid)], 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium butyrate, 0.1 mM PMSF, 2.5 mM DTT (pH 6.8)} containing 75 mM NaCl and were digested with 20 U of micrococcal nuclease per mg of chromatin for 4 min at 37°C. The micrococcal nuclease reaction was quenched with EDTA to a final concentration of 5 mM and digested nuclei were centrifuged at 5000 x g for 5 min at 4°C. The supernatant containing the highly acetylated nucleosomes was collected and purified by dialysis against buffer C (10 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2$, 350 mM NaCl, 10 mM sodium butyrate, 2.5 mM DTT and 0.1 mM PMSF) (pH 7.5) overnight at 4°C. Linker histones and other non-histone associated proteins were removed from the chromatin fragments by incubation with 20 mg of carboxymethyl-Sephadex per mg of chromatin with gentle stirring for 3 h at 4°C. The histones were than purified on a Sepharose CL-4B column as described in section 2.11.1.1 except that the elution buffer also contained 5 mM sodium butyrate. The preparation was quantitated by measuring the absorbance at 230 nm and was concentrated to 10 mg/ml using a centricon-30 concentrating cell (Amicon), diluted to 5 mg/ml with glycerol and stored at -20°C.

2.11.1.5 Preparation of purified chicken histone H2A-H2B dimers

Histone H2A-2B dimers were purified from chicken erythrocyte nuclei using the method of Simon and Felsenfeld (1979). 3 ml of sheared chicken chromatin (section 2.1.4) was mixed with 25 ml of loading buffer (0.63 M NaCl, 0.1 M potassium phosphate pH 6. 7, 0.1 mM DTT, 10 μg/ml PMSF) and loaded onto a 5 ml hydroxylapatite column that had been equilibrated with the same buffer at 4 °C. The column was washed with a further 25

ml of loading buffer before elution of the H2A-H2B dimers with running buffer (0.93 M NaCl, 0.1 M potassium phosphate pH 6.7, 0.1 mM DTT, 10 µg/ml PMSF). Each fraction was assayed for concentration and peak fractions were dialysed overnight at 4 °C against the dialysis buffer (200 mM NaCl, 20 mM HEPES pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10 µg/ml PMSF, 2 µg/ml leupeptin and 2µg/ml pepstatin. Aliquots of the dialyzed fractions were then analysed by SDS-PAGE and silver stained to determine the peak fractions. Desired fractions were pooled and concentrated using micro-concentrators (Amicon, 3000 Dalton MWCO). The final combined volume from the various fractions was aliquoted into 10 to 15 µl and stored at -70 °C.

2.11.1.6 Preparation of purified chicken and HeLa histone H3-H4 tetramers

In order to purify H3-H4 tetramers, chicken long chromatin was prepared as for H2A/H2B dimer isolation (section 2.11.1.5). After elution of the H2A/H2B fractions, the column was washed with 6 column volumes of buffer B (0.93 M NaCl, 0.1 M potassium phosphate pH 6.7, 0.1 mM DTT, 10 µg/ml PMSF). The column was washed with 2 column volumes of buffer B (containing 1.2 M NaCl) before elution of H4/H4 with buffer B containing 2 M NaCl. Peak protein containing fractions were pooled and dialysed against H3-H4 buffer (400 mM NaCl, 10 mM HEPES pH 7.5, 1 mM DTT, 10 µg/ml PMSF, 2 µg/ml leupeptin and 2µg/ml pepstatin).

2.11.1.7 Preparation of recombinant Nucleosome Assembly Factor-1 (NAP-1) and N1/N2 in *E.coli*

a. Construction of expression vectors

T7 tagged Yeast NAP-1 was supplied as pTN2, a kind gift from T. Fugii-Nakata (Fugii-Nakata *et al.*, 1992). The cDNA for NAP-1 was sub-cloned into the bacterial expression plasmid (pQE vector, Qiagen) so that the sequence was in frame with the 6 x His N-terminal tag. The coding sequence for NAP-1 was excised from pTN2 with *Nde* I. This fragment was end-filled and blunt-end ligated into *Sma*I digested and de-phosphorylated pQE30 (Qaigen) according to section 2.1. The resulting plasmid was termed pQE-NAP-1.

b. Protein expression

NAP-1 was expressed in *E.coli* and denatured in 8M urea before purification by ~~nickle~~ nickel column chromatography using a method modified from Thanos and Maniatis (1996). The pQE-NAP-1 expression plasmid was transformed into M15(rep4) (Qaigen) competent cells according to section 2.1.11 and 2.1.12, plated on selective LB/agar plates (100 µg/ml ampicillan, 25 µg/ml kanamycin) and incubated overnight at 37°C. An individual colony was used to inoculate a 10 ml overnight LB culture (ampicillin 100 ug/ml, 25 µg/ml kanamycin) and grown overnight at 37°C, 260rpm. 500 ml of LB (100 µg/ml ampicillan, 25 µg/ml kanamycin) was inoculated with the overnight culture and grown at 37 °C 260 rpm until the absorbance of the culture at 600 nm reached between 0.5 and 0.7. Protein expression was induced by adding IPTG to a final concentration of 1 mM and the culture was incubated a further 4 hours at 37 °C. The cell pellet was collected by centrifugation at 4000 rpm for 15 min at 4°C in GSA bottles in a RC5B Sorvall Centrifuge and the bacterial pellet was frozen at -70°C overnight.

c. Purification of recombinant NAP-1

The frozen bacterial pellet was thawed and resuspended in 20 ml buffer A (8 M urea, 100 mM Sodium Phosphate, pH 8, 10 mM Tris-HCl pH 8, 10 mM imidazole, 0.5 mM PMSF, 10 mM 2-ME, 10% (v/v) glycerol), transferred to SS-34 tubes and incubated for 20 minutes at rt. Cellular debris were pelleted by centrifugation in a SS-34 rotor at 10 000 rpm at rt. The cleared supernatant was mixed with 1ml of Ni-NTA resin (Qaigen), washed and equilibrated as described in section (2.3.1.2b), however the buffer used was buffer A. This mixture was incubated on a rotating platform at rt for 4 hrs and pelleted by centrifugation at 1000 rpm for 5 min at 4°C in a Beckman Allegra 6G clinical centrifuge. The pelleted resin was resuspended in 2 ml of buffer A (20 mM imidazole) and transferred to a 10 ml poly-prep chromatography column (Bio-Rad). The column was washed with 20 ml of buffer A (containing 20 mM imidazole) before elution with buffer A (100 mM imidazole). 300 µl fractions were collected from the protein elution and were analysed on SDS-PAGE. Peak protein containing fractions were pooled and renatured by step-wise dialysis.

Pooled fractions were transferred to Slide-A-Lyser cassettes (Pierce) and dialysed against 500 ml dialysis buffer (6 M urea, 500 mM NaCl, 20 mM HEPES pH 7.9, 10% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF, 0.1% NP-40) for 2 hrs at 4°C. 250 ml of dialysis buffer (which lacks urea) was added and the dialysis was continued overnight so that the final urea concentration was 4 M. The volume was reduced to 400 ml and 133 ml of buffer was added (urea concentration is reduced to 3 M) and the dialysis continued for 2-3 hrs. 267 ml buffer was added and dialysed for an additional 3 hrs (2 M urea). The dialysis volume was reduced to 400 ml and an equal volume of buffer was added to reduce the urea concentration to 1 M and continued for a further 3 hrs. This step was repeated over-night (0.5 M urea). The dialysis cassettes were transferred to a clean beaker and dialysed against buffer containing no urea, twice, with one buffer change for two hours each time. After removing the insoluble material by centrifugation at 14000 X g at 4 °C, the proteins were dialysed against EB storage buffer (1 mM EGTA, 20 mM HEPES pH7.5, 1.5 mM $MgCl_2$, 10% (v/v) glycerol, 50 mM DTT, 10 mM 2-ME, 60 mM KCl, 10 µg/ml PMSF, 2 µg/ml pepstatin, 2µg/ml leupeptin) and stored in aliquots at -70°C.

2.11.2 Nucleosome assembly

2.11.2.1 Nucleosome assembly by histone octomer transfer

The histone octamer transfer method was used to reconstitute 147 to 180 bp fragments of DNA into a single nucleosome. The assembly of nucleosomes was carried out essentially as described by Adams and Workman (1995). Approximately 25 ng of end labelled DNA fragment (3 to 3.5 x 10^5 cpm) (section 2.4.3) was mixed with H1 depleted oligonucleosomes from chicken erythrocytes (section 2.11.1.1) or acetylated chromatin from HeLa cells (section 2.11.1.4) at 1 M NaCl in a 20 µl reaction volume. Following incubation at 37°C for 30 minutes, the reaction was diluted (5 steps) to 0.2 M NaCl in 100 µl final volume with 10 mM HEPES pH 7.5, 1mM EDTA with a 25 minute incubation at 30°C between each dilution step. A final 2-fold dilution step to 0.1 mM NaCl (200 µl final volume) with buffer containing 10 mM HEPES, 1mM EDTA, 0.1% (v/v) NP-40, 20 % (v/v) glycerol and 5 mM DTT was performed. This was followed by the addition of $MgCl_2$

to a final concentration of 2.5 mM before incubation on ice for 30 min followed by centrifugation at 16000 x g for 30 min to remove the aggregated chromatin (Schwarz and Hansen, 1994). The supernatant was transferred to a new tube and 5 or 10 µl (comprising 0.5-1 ng labelled fragment that yielded 15000 cpm for each binding reaction) was used for the nucleosome binding assays. For mock reconstitution, TE buffer was substituted for the volume of oligonucleosomes used. The assembled nucleosomes were stored at 4 °C for up to 2 weeks.

2.11.2.2 Nucleosome assembly using the Xenopus S-150 extract

A standard nucleosome assembly using the *Xenopus* S-150 extract (section 2.11.1.2) was carried out in a final volume of 25 µl. The reaction consisted of the S-150 extract, 100 ng of plasmid DNA, 1 ng/µl creatine kinase, 1.5 mM ATP, 100 mM creatine phosphate, 1.5 mM $MgCl_2$ in 1 x EB buffer (1 mM EGTA, 20 mM HEPES pH 7.0, 5 mM KCl, 10 % (v/v) glycerol, 0.5 mM DTT and 10 mM β-glycerol-phosphate). All incubations were carried out at 27°C for 4 hours. Because the extent of DNA supercoiling observed at a given S-150 amount varied among different preparations of S-150 extract, the amount of S-150 was determined by titration against the DNA for each preparation. For mock reconstitutions, the S-150 extract was omitted and replaced with NaCl to a final concentration of 19.2 mM in the reaction.

2.11.2.3 Nucleosome assembly using the Xenopus N1/N2(H3,H4) extract

The assembly of nucleosomes was carried out essentially as described by Tremethick and Frommer (1992). The standard nucleosome assembly reaction was performed in a 32 µl final volume which consisted of N1/N2(H3,H4) complex, H2A/H2B dimer, 300 ng plasmid DNA, 80 mM NaCl and 2.6 mM EDTA in 1 x EB buffer (1 mM EGTA, 20 mM HEPES pH 7.0, 5 mM KCl, 10 % (v/v) glycerol, 0.5 mM DTT and 10 mM β-glycerol-phosphate). The reactions were incubated at 37°C for 4 hours. Because the level of DNA supercoiling observed at a given histone/DNA ratio varied among different preparations of

N1/N2(H3,H4) complexes and H2A/H2B dimers, the levels of both preparations were titrated against each other to yield optimum supercoiling as seen on a 1% agarose gel in the presence of topoisomerase I. Typically the levels of N1/N2(H3,H4) complexes varied between 10 and 25 µl and the level of H2A-H2B dimers varied between 1 and 5 µl among different preparations. For mock reconstitutions, the histones were omitted and the histone buffers were used instead.

2.11.2.4 Nucleosome assembly using NAP-1

Standard reconstitution reactions involving NAP-1were performed in 12 µl final volume which consisted of recombinant NAP-1 (Section 2.11.1.4), purified chicken H3-H4 tetramer (section 2.11.1.6) and purified H2A/H2B dimer (section 2.11.2.5) with 300 ng plasmid DNA, 2 U topoisomerase I (Promega) and 1.5 mM ATP in 1 x EB buffer (1 mM EGTA, 20 mM HEPES pH 7.0, 5 mM KCl, 10 % (v/v) glycerol, 0.5 mM DTT and 10 mM β-glycerol-phosphate). The reactions were incubated for 4 hours at 30 °C. Because the level of DNA supercoiling observed at a given histone/DNA ratio varied among different preparations of H3-H4, H2A/H2B and NAP-1, the level of each component was determined by titration so that optimum levels of supercoiling were achieved for each preparation. Typically, 0.5-1 µl of H3-H4 tetramer, 1.5-3 µl of H2A/H2B and 1-4 µl NAP-1 were used in the reconstitution reactions. For mock reconstitutions, the histones and/or NAP-1 were omitted and the histone or NAP-1 buffers were used instead.

2.12 Cell Culture

The human T cell leukaemic line Jurkat was supplied by Dr A. Hapel (JCSMR). Jurkat cells were maintained at a density of 5 x 10^5 cells/ml in RPMI-1640 containing 10% foetal calf serum (Commonwealth Serum Laboratories [CSL]), supplemented with 60 µg/ml benzylpenicillin (CSL), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (CSL). The cells were grown in 5% CO_2 and 95% relative humidity.

2.12.1 Plasmid constructs for transfection

The same 700 bp HTLV-I LTR fragment from pUR3-CAT used for *in vitro* transcription (section 2.5) was excised using *Hind*III and *Xho*I. The 700 bp HTLV-I LTR fragment was sub-cloned into pXP1-G, a luciferase-z reporter construct (Nordeen, 1988), digested with the same enzymes. The luciferase reporter construct created was termed pLucHTLV. The anti-sense HMG-I(Y) construct (pRcCMVIGMH) was supplied by Dr F. Shannon (JCSMR) and is described in Himes *et al.,* (1996). The *tax-1* plasmid BC15S, was obtained from Dr J. Rosenblatt (UCLA, USA). The plasmid was prepared by cloning the *tax* cDNA into BC12 (Cullen, 1986) which contains the cytomegalovirus–1E promoter and the rat preproinsulin II genomic sequence.

2.12.2 Transient transfection

Cell transfection was achieved using a activated-dendrimer reagent (Superfect, Qiagen). Transfections were performed 25 cm^2 flasks using 1.5 x 10^6 cells in a total volume of 2 ml. Cells were transfected with 1 µg of pLuc-HTLV(reporter) and up to 2 µg of the HMG-I anti-sense construct pRcCMVIGMH. DNA was made up to 3 µg total with pRcCMV vector lacking the anti-sense HMG-I insert. Transfections were carried out according to the manufactures protocol using a DNA (µg): Superfect (µl) ratio of 1:5, with the exception that the reporter construct was complexed with the Superfect separately from the other DNA before addition to the cells. This minimised variations in expression between transfections. DNA-Superfect complexes were added to the cells for 4 hrs after which an additional 3 ml of media was added and incubated for a further 18 hrs. Where cells were stimulated with Phorbol-12-myristate-13-acetate (PMA), PMA (32 nM) was added to the media and cultured for an additional 9 hours.

2.12.3 Luciferase assay

Cells were harvested by centrifugation, resuspended and lysed in 200 µl cell lysis buffer (0.1 M K_2HPO_4, pH 7.8, 1 mM EDTA pH 8.0, 0.2 mM DTT, 1% (v/v) triton X-100). Cells debris was pelleted by centrifugation and the total protein concentration of the supernatant was determined using the BioRad protein assay and normalised for total protein. Typically, 50 or 100 µg protein was loaded onto 96 well plates combined with 200

µl of assay buffer (100 mM K_2HPO_4, pH 7.8, 2 mM DTT, 10 mM $MgSO_4$, 320 mM CoA, 500 mM ATP). To begin the light reaction, 40 µl of D-luciferin (in 5 mM K_2HPO_4 buffer) was added to each well. The light reaction was read on a Topcounter (Packard).

CHAPTER 3:

Recombinant synthesis and characterisation of the major HTLV-I LTR interacting proteins

3.1 Introduction

An important step in the regulation of gene expression is the binding of sequence specific transcription factors to their cognate sites on DNA. Once bound, the transcription factor can activate transcription by the recruitment of cofactors, or by direct interaction with the basal transcriptional machinery. *In vivo*, transcription factors must gain access to DNA contained in nucleosomes. However, in order to understand the effect of nucleosomes on factor binding to DNA, it is necessary to first characterise the interaction with the transcription factors on naked DNA.

It is well established in the literature that the HTLV-I encoded protein Tax is critical for high level activation of gene expression from the virus (Chen *et al.*, 1985). This conclusion has been reached largely from tissue culture studies where transfected *tax* can activate the expression of HTLV-I LTR reporter constructs (Cullen, 1986). However, the mechanism by which Tax activates transcription form the LTR is not completely understood. Tax is thought to interact indirectly with DNA through cellular transcription factors and numerous studies have identified members of the CREB/ATF transcription factor family as the principal cellular factors involved. Of the ATF/CREB family, CREB is the best characterised with respect to mediating HTLV-I transcription. Figures 1.9 and 1.11 show a schematic representation of the Tax and CREB proteins and their sites of interaction with DNA and other factors.

In this chapter the major HTLV-I interacting proteins Tax and CREB were synthesised as recombinant proteins utilising baculovirus and bacterial expression systems respectively, and purified by affinity column chromatography. The binding of these proteins to naked DNA was assessed by gel mobility shift and DNase I footprinting assays for two reasons. Firstly, to ascertain whether these recombinant proteins are functional, and secondly to characterise their interactions both individually, and in combination with naked DNA as an important first step in understanding the control of HTLV-I transcription.

On the HTLV-I LTR, three imperfect 21 bp repeat elements, termed Tax responsive elements (TREs), are the site for CREB and Tax interaction. The repeats consist of a central element TGACGTC/A flanked by GC rich sequence (see figure 1.8). CREB binds to the central sequence while Tax interacts directly with CREB and with the GC rich flanking sequences of the TRE-1 (Kimzey and Dynan, 1998). The promoter of the somatostatin gene, also contains the central core sequence but differs by one base pair. In addition, the somatostatin promoter does not contain the GC rich flanking sequences (figure 1.8). Although this promoter is CREB responsive, Tax has no effect on transcriptional activity (Kwok *et al.*, 1996). Thus, the somatostatin promoter provides an excellent control for the effect of Tax on the HTLV-I LTR. The probes used in this study to characterise the binding of the above proteins were derived directly from the 3' regulatory region of the HTLV-I LTR and from the promoter of the cellular somatostatin gene (see table 2.3 and figure 2.1). For the majority of studies in this chapter a 147 bp HTLV-I probe termed H147(M) and a 180 bp somatostatin probe termed S180 were utilised. These probes were designed such that the core protein binding regions are positioned at the center of the DNA. The size of the probes is specifically for their assembly into a single nucleosome (Chapter 4). However, to investigate specific regions of sequence smaller 30 bp probes were utilised (table 2.2).

At the commencement of this study, CBP had been characterised as an important co-factor for CREB function (Kwok *et al.*, 1994). Therefore in this study, the original intention was to investigate whether CBP has an important role in HTLV-I transcription. CBP, a very large protein consisting of over 2400 amino acids, has many protein binding and functional domains, and has been shown to act as a cofactor for many diverse transcription factors including CREB, nuclear hormone receptors, c-Jun, c-Fos, and MyoD (reviewed in Goodman and Smolik, 2000). A schematic map of CBP including the known functional domains and factor binding sites is shown in figure 1.7. The identification of CBP as an acetylase of histone substrates (at the early stages of this work), indicated that CBP may contribute to transcriptional regulation via the targeted acetylation of chromatin (Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996). Furthermore, the ability of CBP to also acetylate non-histone substrates indicates that there is a high level of complexity involved with the mechanism of CBP mediated transcriptional control. Much of the interaction between CBP and other factors, and acetylation activity, has been examined using truncated forms of CBP (see for example Giebler *et al.*, 1997). However, the true function of full length CBP may involve interaction between the various domains. There were no reports of full length CBP in the literature at the commencement of this study. Therefore, in this

chapter, full length CBP was synthesised using the baculovirus system and purified using Nickel chromatography. Recombinant CBP was then tested for function by assessing its ability to acetylate histones and non-histone substrates. As CBP has also been shown to acetylate other transcription factors, the ability of full length CBP to acetylate other factors related to HTLV-I was examined.

The regulation of transcription is a complex process and one important, but recently identified element of control is the post translational modification of non-histone proteins. These modifications include acetylation and phosphorylation. Although identified as being involved in transcription, the mechanism by which these modifications affect transcription are unclear. In this chapter the effect of some post-translational modifications on the binding properties of the major HTLV-I LTR interacting proteins are analysed. The combination of the use of natural promoters and full length proteins, in addition to assessing the effect of modifications of these proteins, represents an extensive approach to investigating the initial steps of HTLV-I transcription.

3.2 Results

3.2.1 Synthesis and characterisation of recombinant CREB

3.2.1.1 Expression and purification of recombinant CREB

Rat CREB cDNA (pET-CREB) was expressed in *E. coli* as described in the methods (section 2.3.1.1). As a control for the expression and purification process, the pET plasmid without CREB cDNA was expressed and purified in parallel with CREB. The resulting preparation was termed mock CREB. Figure 3.1 (panel A) shows the presence of a 45 kDa CREB band after IPTG induction in the cells transformed with pET-CREB whereas, there is no band present in the mock CREB lane (compare lanes 1 and 2). The first step in the purification of CREB was performed according to the method of Loriaux*et al.* (1993) and involved sonication of the cell lysate, removal of the insoluble material by centrifugation and the heating the soluble fraction at 70°C. This step was successful in the partial purification of CREB only (lane 4). Therefore, it was necessary to refine this method to further purify CREB. As CREB is well characterised as a DNA binding protein, the heated CREB fraction was purified by affinity chromatography using phosphocellulose (section

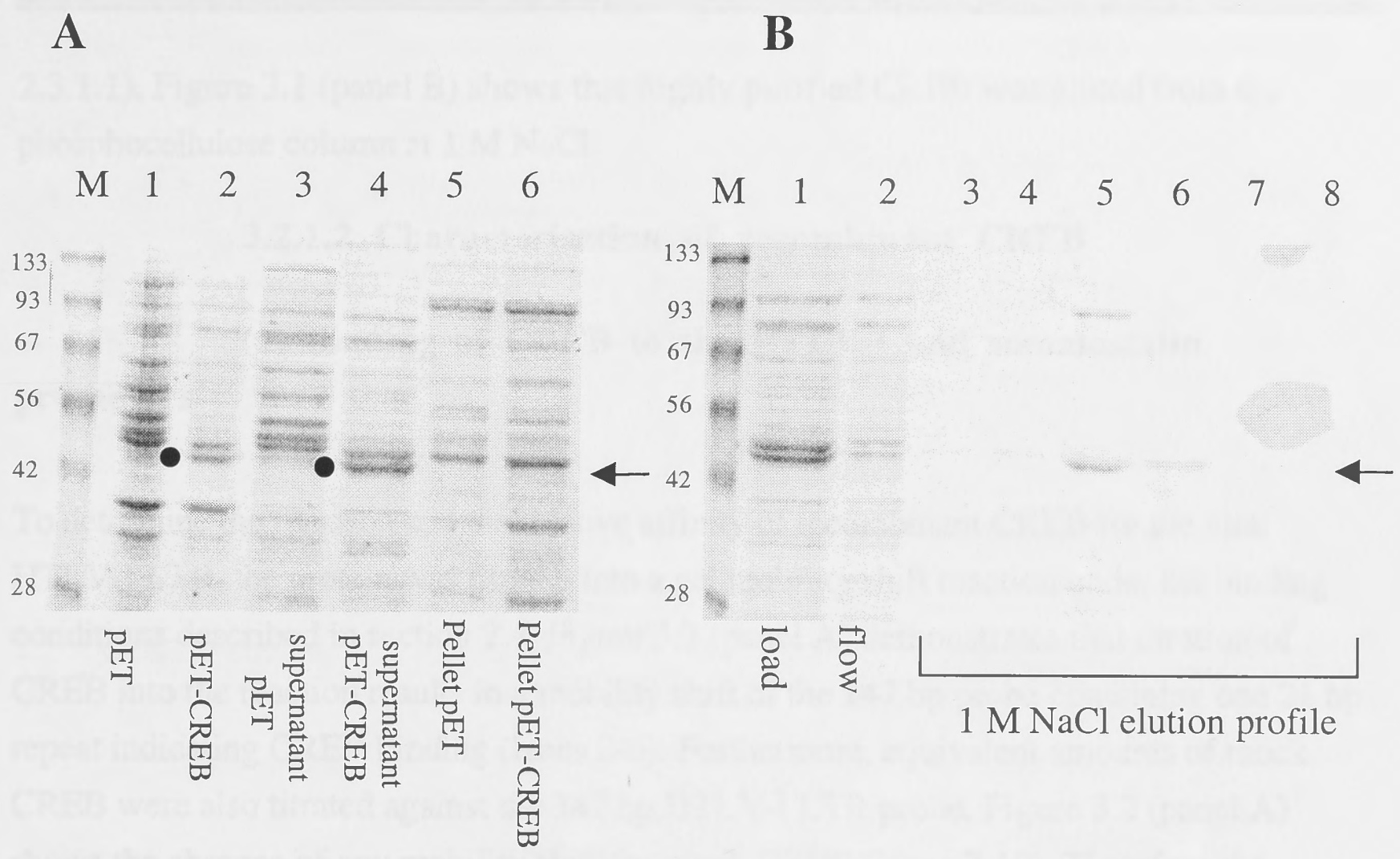


Figure 3.1: Expression and purification of CREB in *E. coli.*
10% SDS-PAGE gels visualised by Coomassie brilliant blue staining of the purification process **A**. Preliminary purification: Protein lysates from the mock CREB and CREB preparations (lanes 1 and 2 respectively); supernatant resulting from centrifugation after heat precipitation of the mock CREB and CREB preparations (lanes 2 and 4); pellets from the same centrifugation step (lanes 5 and 6 for mock CREB and CREB respectively). CREB is indicated by a dot. **B**. Final purification: The preparation shown in A was loaded onto a phosphocellulose column and the column flow through (lanes 1 and 2) and the profile of the 1 M NaCl elution (lanes 2-8) are shown. Molecular weight standards (kDa) are shown at the left of each figure.

2.3.1.1). Figure 3.1 (panel B) shows that highly purified CREB was eluted from the phosphocellulose column at 1 M NaCl.

3.2.1.2 Characterisation of recombinant CREB

(a) Binding of CREB to the HTLV-I and somatostatin promoters

To determine the function and the relative affinity of recombinant CREB for the viral HTLV-I LTR, the protein was titrated into a gel mobility shift reaction under the binding conditions described in section 2.4. Figure 3.2 (panel A) demonstrates that titration of CREB into the reaction results in a mobility shift of the 147 bp probe containing one 21 bp repeat indicating CREB binding (lanes 2-5). Furthermore, equivalent amounts of mock CREB were also titrated against the 147 bp HTLV-I LTR probe. Figure 3.2 (panel A) shows the absence of any mobility shift for mock CREB (lanes 7-10). Therefore the mobility shift in lanes 2-5 can be attributed to CREB. A band of faster mobility than the CREB/DNA complex is apparent in all the CREB mobility shift assays in this work. We believe that this complex is a break-down product from the CREB purification process.

To determine the exact site of CREB binding on the 147 bp probe, DNAse I footprinting was carried out using identical binding conditions to the gel mobility shift (section 2.4.3). Figure 3.2 (panel B) shows a CREB footprint on the 147 bp probe at the TRE element. The detection of a CREB footprint at a site known to bind CREB indicates that CREB binds specifically to the HTLV-I LTR under the conditions used in this study.

A comparison between the binding affinities of CREB for the HTLV-I LTR and the somatostatin CRE consensus site was also conducted and is shown in figure 3.3. The probes used throughout this study were quantitated by counting and diluted to 25000 cpm for use in gel shift assays (see section 2.4.2). This quantitation ensures that an equal amount of each different probe is added to the mobility shift reactions. Therefore the intensity of the mobility shifted band is directly related to the degree of binding. Figure 3.3 shows that CREB also binds the consensus CRE (panel B) with a higher affinity (approx. 5-fold) than for the HTLV-I TRE (panel A).

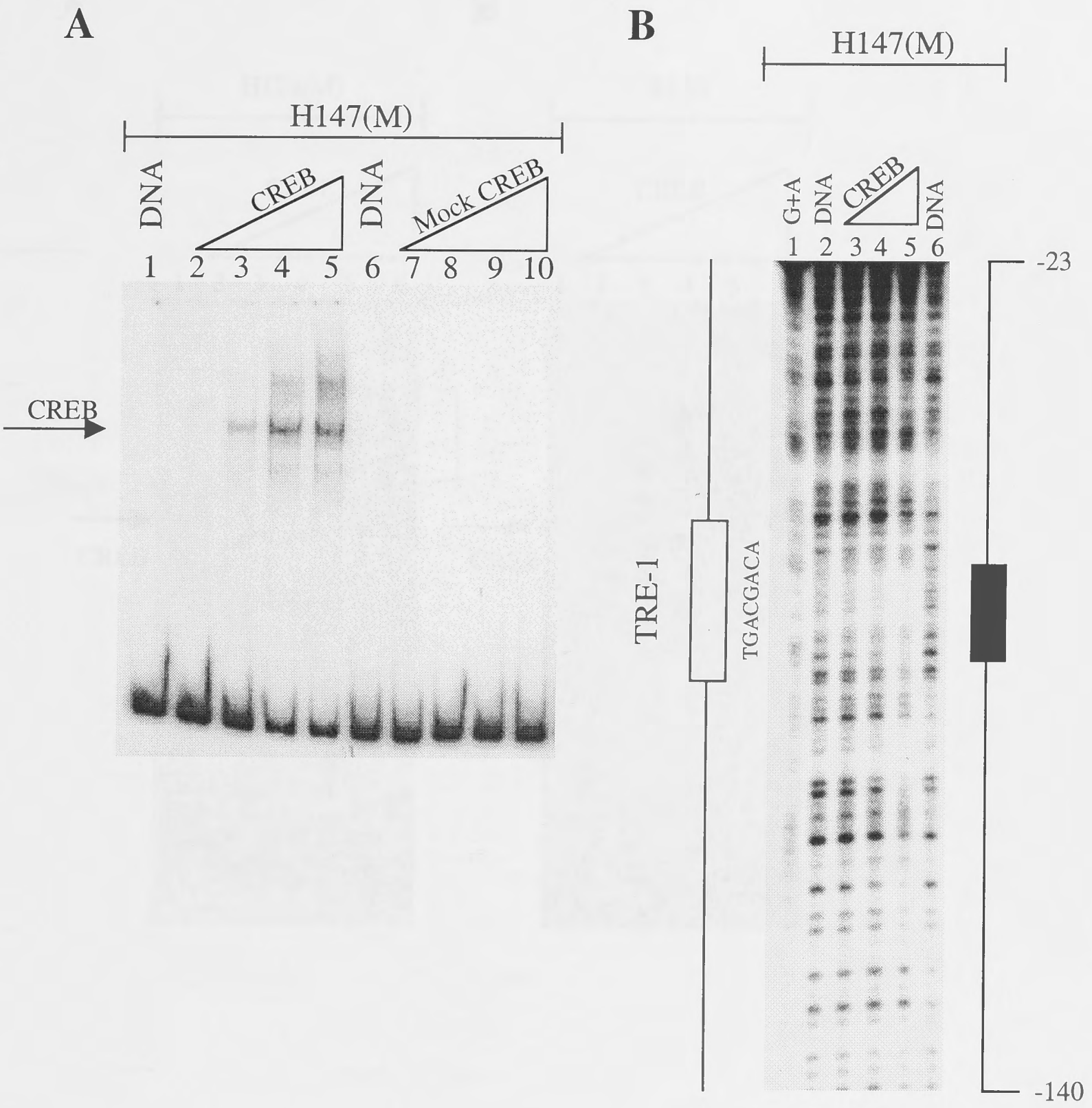


Figure 3.2: Binding of CREB to the HTLV-I promoter.
A. A Gel mobility shift reaction of CREB and mock CREB bound to the H147(M) probe: Lanes 1 and 6 contain DNA alone. Lanes 3-5 contain 1, 2 and 3 µl of CREB (1µg, 2µg and 3µg). Lanes 7-10 contain 1, 2 and 3 µl of mock CREB. **B**. DNase I footprinting assay of CREB binding to the same promoter. Lane 1: G+A ladder; Lanes 2 and 4: Naked DNA; lanes 3-5: 1, 2 and 3 µg of CREB. The region of DNase I protection is indicated by a solid rectangle. The Tax responsive element (TRE) is shown on the right. Sequence is numbered relative to the start site of transcription.

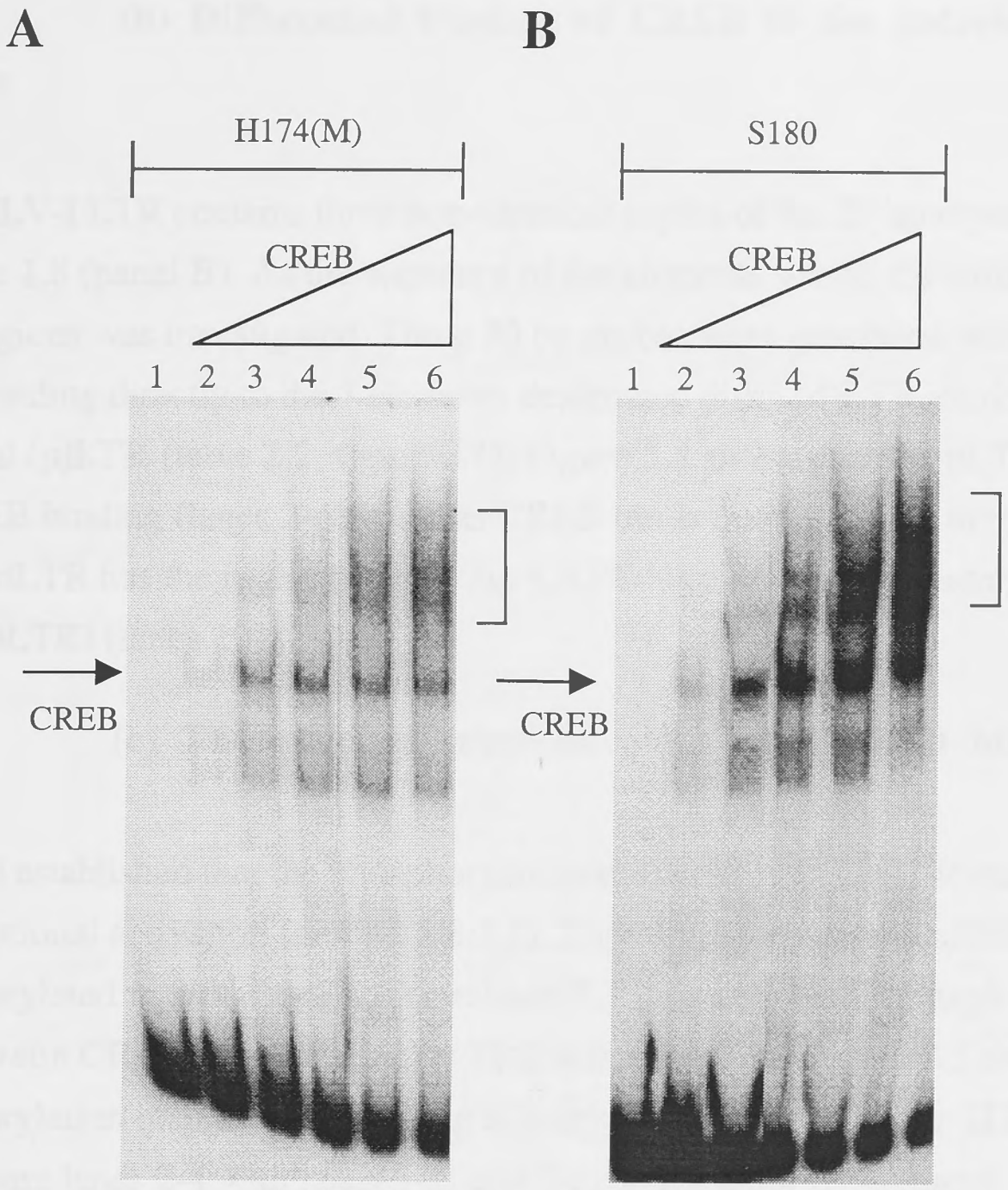


Figure 3.3: Comparison between the binding affinities of CREB for the HTLV-I and somatostatin probes. A. A gel mobility shift of CREB bound to the H147 probe: Lane 1: Naked DNA; Lanes 2-6: 0.5, 1, 2, 3 and 4 μg of CREB respectively. **B.** A gel mobility shift of CREB bound to the somatostatin probe: Lanes as for panel A. The position of the CREB/DNA complex is marked by an arrow. Oligomeric complexes are marked by a bracket.

(b) Differential binding of CREB to the individual 21 bp repeats

The HTLV-I LTR contains three non-identical copies of the 21 bp repeat element as shown in figure 1.8 (panel B). As the sequence of the elements varies, the affinity of CREB for these regions was investigated. Three 30 bp probes were generated with sequence corresponding directly to the 3 elements designated distal (d)LTR, middle (m)LTR and proximal (p)LTR (table 2.2, figure 2.1). Figure 3.4 shows that the pLTR has low affinity for CREB binding (lanes 2-4) whereas CREB binds more strongly to the mLTR (lanes 6-8). The dLTR has the highest affinity for CREB binding (approximately 5 fold higher that for the pLTR) (lanes 10-12).

(c) The effect of phosphorylation on CREB binding

It is well established that the phosphorylation of CREB is critical for maximal transcriptional activation (section 1.8.2.1). Therefore, the binding affinity of CREB phosphorylated *in vitro* by PKA (methods 2.3.1.1a), for both the high affinity somatostatin CRE and lower affinity TRE was examined. Figure 3.5 shows that phosphorylation increases the binding affinity of CREB for both the HTLV-I TRE (panel A, compare lanes 2-4 with lanes 5-7) and the somatostatin CRE (panel B, compare lanes 2 and 3 to lanes 4 and 5). Interestingly phosphorylation of CREB also resulted in a significant change in the mobility of the CREB band shift such that the complex ran more slowly (Figure 3.5, panel A, compare lanes 3 and 4 to lanes 6 and 7). Therefore, we conclude that based on DNA binding studies, recombinant CREB is functional.

3.2.2 Synthesis and characterisation of recombinant N-Tax expressed in baculovirus

3.2.2.1 Expression and purification

(a) The construction of recombinant N-Tax baculovirus

The baculovirus protein expression system was chosen to express Tax as it is capable of a variety of eukaryotic post-transcriptional modifications, including glycosylation, accurate signal peptide cleavage and phosphorylation. In addition, Tax expressed by the baculovirus system has been previously reported to be correctly post-translationally modified and active

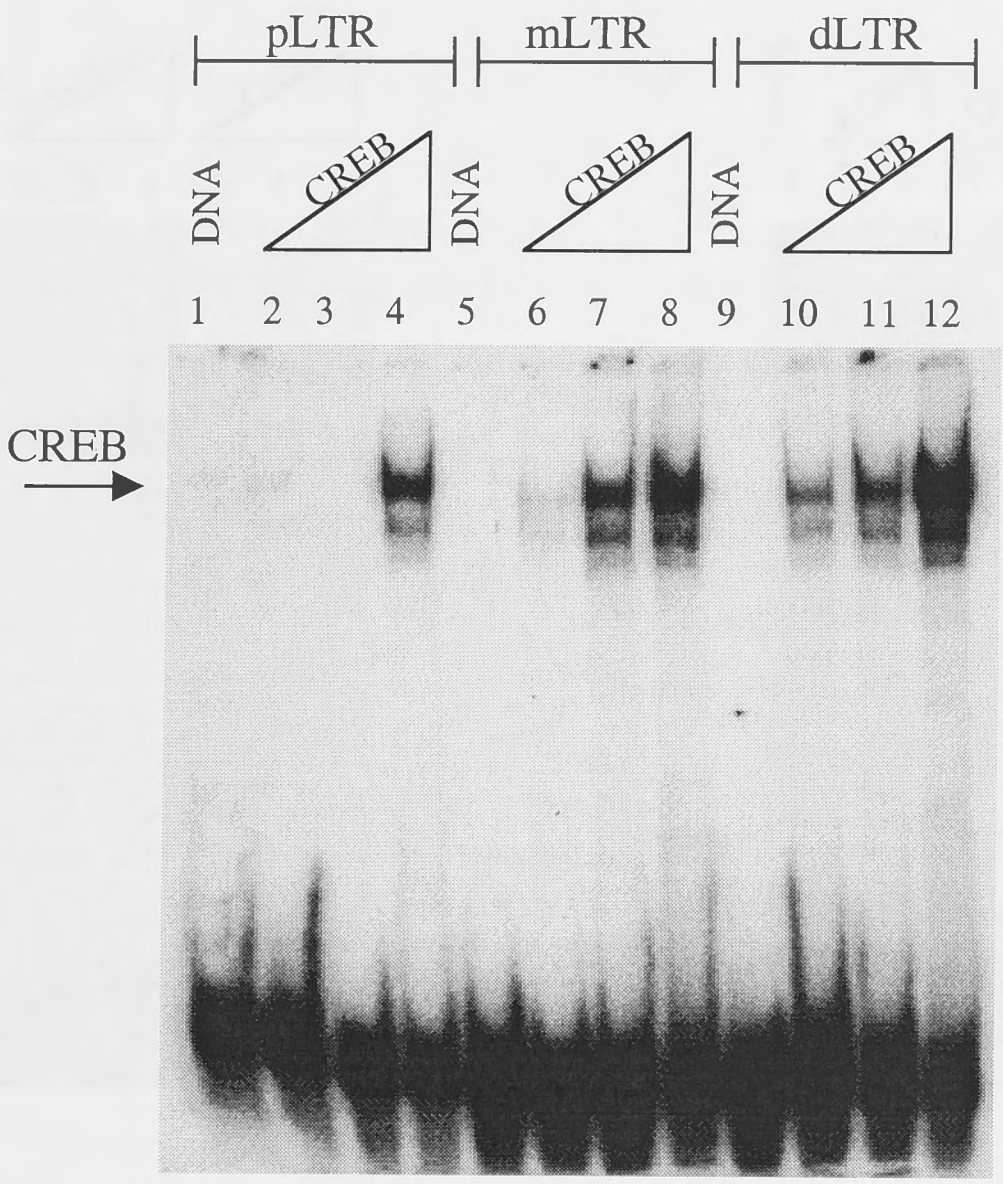


Figure 3.4: Differential binding of CREB to the individual 21 bp repeats. A. Sequence comparison of the three 21 bp Tax responsive elements (TREs) on the HTLV-I LTR. The TRE elements are labelled according to their position, either, proximal (p), medial (m) or distal (d) relative to the TATA box. **B**. Gel mobility shift of increasing concentrations of CREB bound to the three TRE elements on the HTLV-I promoter (see figure 2.1): Lanes 1-4: pLTR; lanes 5-8 mLTR; lanes 9-12; dLTR. Lanes 1, 5 and 10: naked DNA. CREB concentrations are 1 μg (lanes 2, 6 and 10); 2 μg,(lanes 3, 7 and 11); 4 μg (lanes 4, 8 and 12). The CREB/DNA complex is indicated by an arrow.

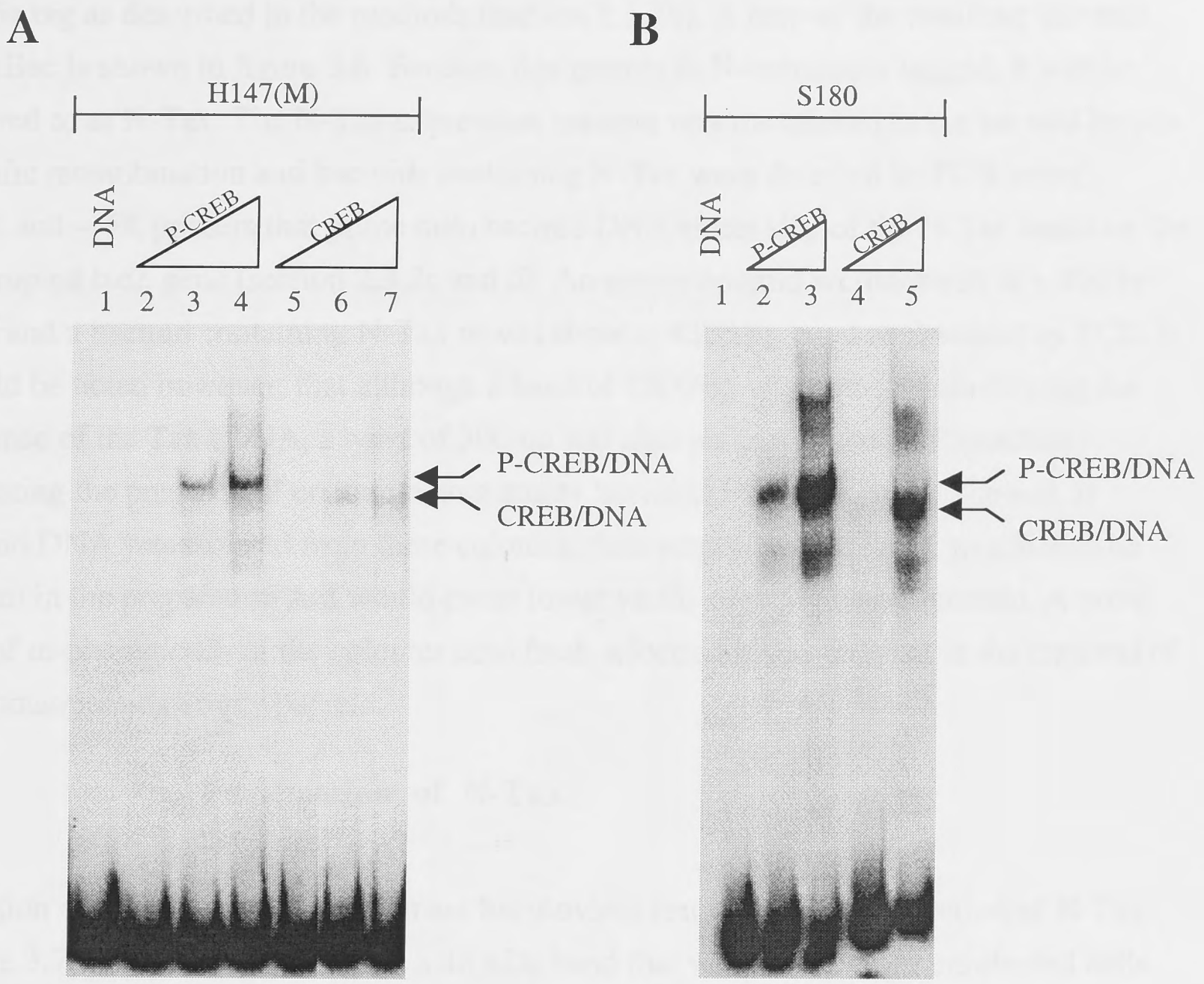


Figure 3.5: Phosphorylated CREB binds with higher affinity than CREB to the HTLV-I and somatostatin probes. A gel mobility shift of CREB and phosphorylated CREB bound to both the HTLV-I and somatostatin probes. **A.** Lane 1: Naked DNA; lanes 2-4 and 6-7: 0.5, 1 and 2 μg of mock phosphorylated and phosphorylated CREB respectively. **B.** Lane 1: Naked DNA; lanes 2-3 and 4-5: 0.5 and 2 μg of phosphorylated and mock phosphorylated CREB respectively. The CREB/DNA and P-CREB/DNA complexes are indicated by arrows.

in vivo (Jeang *et al.*, 1987; Matthews *et al.*, 1992). Tax cDNA derived from a Gal-Tax fusion construct was sub-cloned into the FastBac transfer plasmid 3' from the N-terminal 6XHis tag as described in the methods (section 2.3.2b). A map of the resulting plasmid pTaxBac is shown in figure 3.6. Because this protein is N-terminally tagged, it will be referred to as N-Tax. The N-Tax expression cassette was transferred to the bacmid by site specific recombination and bacmids containing N-Tax were detected by PCR using -40L and -44R primers that prime onto bacmid DNA either side of the N-Tax insert on the interrupted lacZ gene (section 2.3.2c and d). An empty bacmid would result in a 300 bp band and a bacmid containing N-Tax would show a 4300 bp band as detected by PCR. It should be noted however, that although a band of 4300 bp was detected confirming the presence of the Tax cDNA, a band of 300 bp was also present in the PCR reaction indicating the presence of contaminating empty bacmid DNA (results not shown). If bacmid DNA was isolated from these colonies, than empty bacmid DNA would also be present in the preparation and would cause lower yields of recombinant protein. A novel step of re-plating each of the colonies onto fresh selection plates resulted in the removal of the contaminating empty bacmid.

(b) Purification of N-Tax

Infection of Sf9 cells with recombinant baculovirus resulted in the production of N-Tax. Figure 3.7, panel A (lane 2) shows a 48 kDa band that was absent from uninfected cells (lane 1) or cells infected with bacmid only DNA (lane 3). This band was verified as N-Tax by Western analysis (results not shown). Conventional methods for harvesting recombinant proteins from the insect cells were unsuccessful as most of the protein remained in the insoluble portion of the preparations. Therefore a method was developed using sequential salt extraction to solublise N-Tax (section 2.3.2i). The level of N-Tax after each step of the purification process was monitored by Western analysis (results not shown). After lysis of the cell pellet in low salt buffer, the majority of the protein was present in the nuclear fraction. Following extraction in 420 mM NaCl buffer, a proportion of Tax was present in the soluble fraction. This preparation was termed high salt supernatant 1 (HSS1) and was further purified by Nickel affinity chromatography. The insoluble component was further extracted with 840 mM NaCl. The soluble portion from this extraction was termed high salt supernatant 2 (HSS2) and was also purified by column chromatography. However, despite the salt extractions, a portion of N-Tax remained in the insoluble fraction. Purified N-Tax protein, from HSS1 after elution from the Nickel column with 100 mM imidazole is shown in figure 3.7, panel B. In addition, the identity of

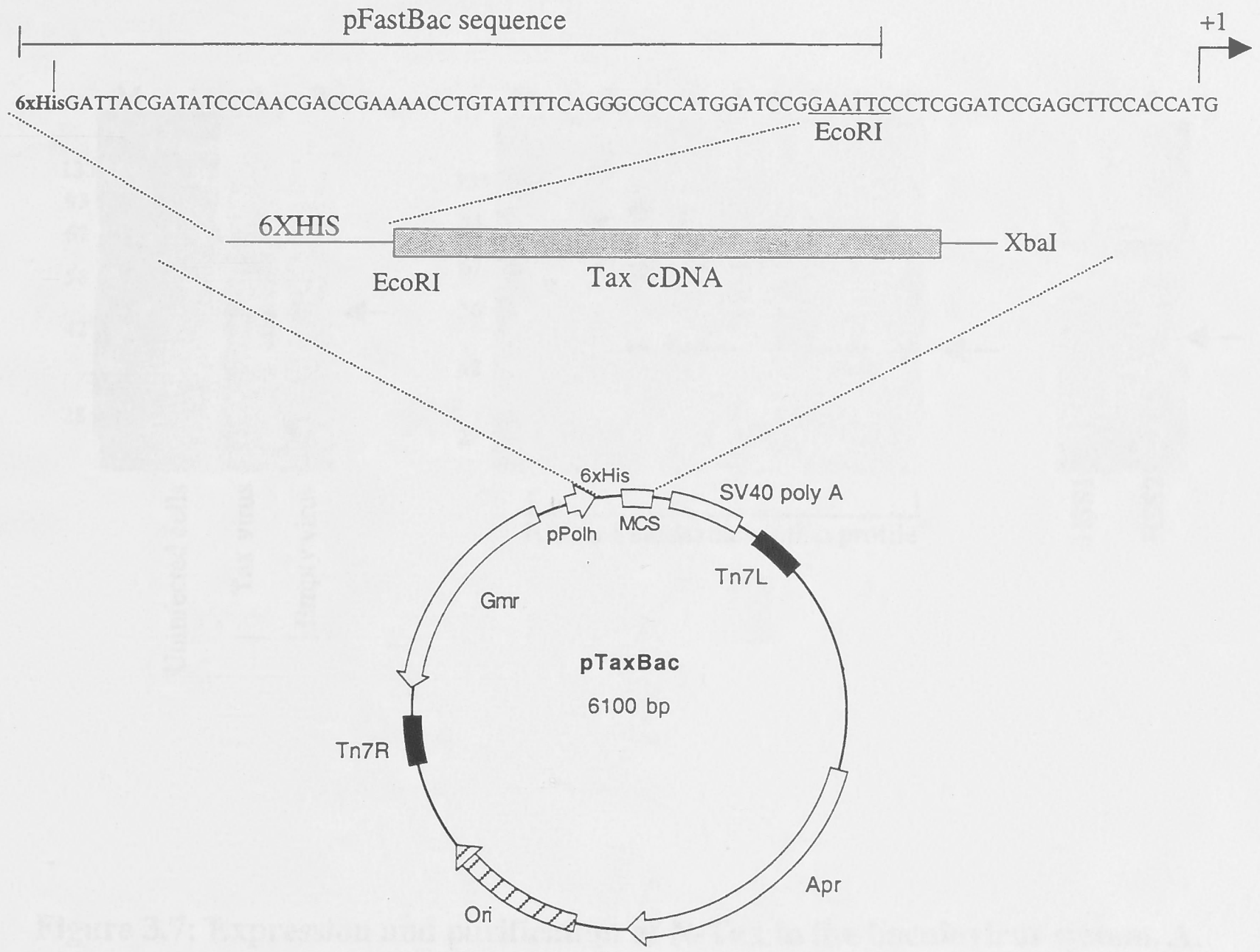


Figure 3.6: Sub-cloning of N-Tax. A schematic plasmid map of the pTax-Bac transfer vector used to create the recombinant N-Tax baculovirus.

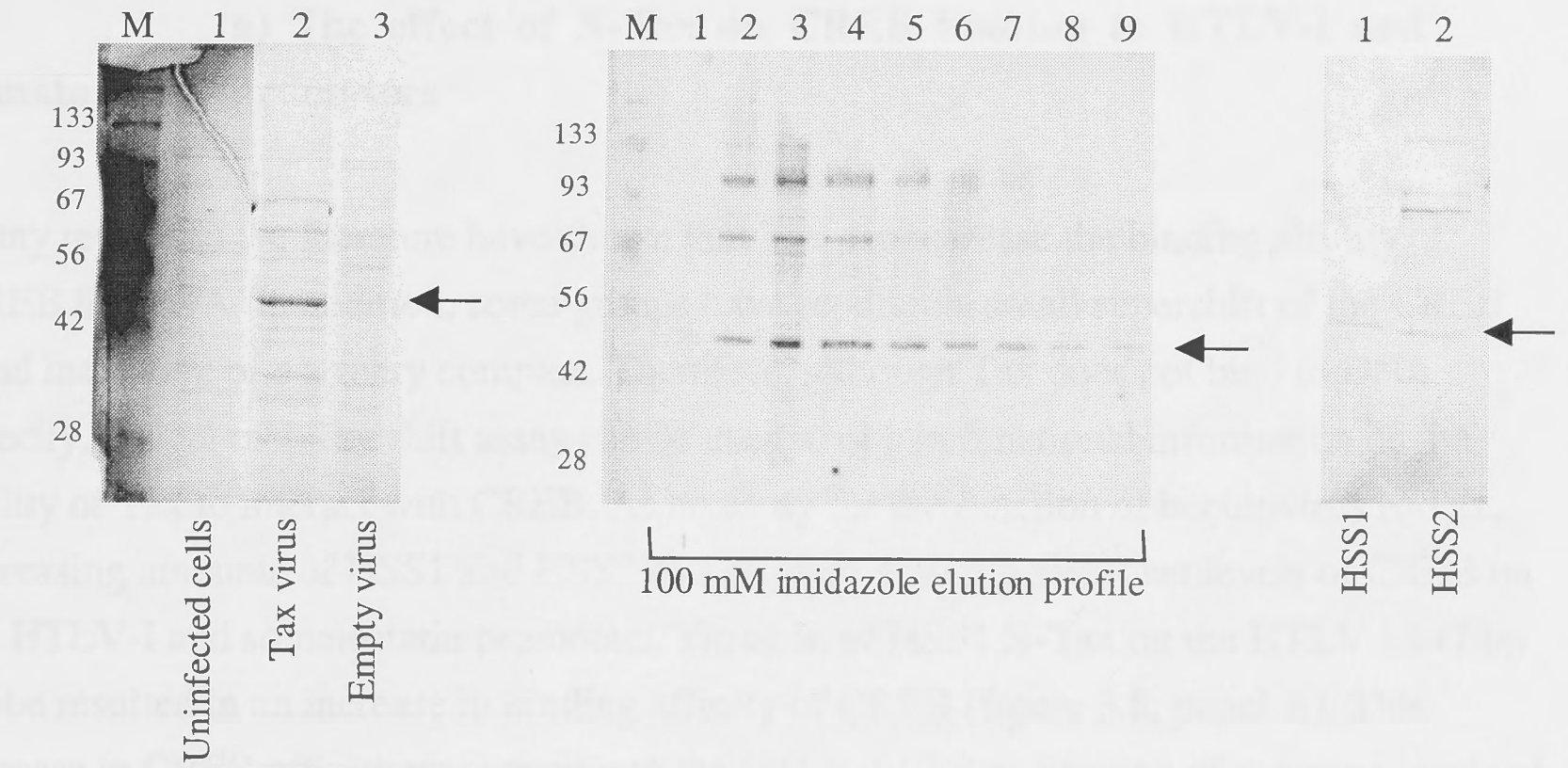


Figure 3.7: Expression and purification of N-Tax in the baculovirus system. A. Expression of N-Tax in Sf-9 cells : Cell lysates from uninfected cells (lane 1); cells infected with the recombinant N-Tax baculovirus (lane 2); cells infected with wild type baculovirus (lane 3) were run on 10% SDS-PAGE and visualised by Coomassie brilliant blue staining. **B**. HSS1 N-Tax preparations were loaded onto a Nickel column and the profile of the 100 mM imidazole elution (lanes 1-9) is shown on a 10% SDS-PAGE gel visualised by silver staining. **C**. Western analysis of HSS1 and HSS2 N-Tax preparations resulting from purification by Nickel chromatography. Lane 1: HSS1 N-Tax preparation; lane 2: HSS2 N-Tax preparation. Molecular weight standards (KDa) are shown at the left of each figure.

the purified protein from both HSS1 and HSS2 preparations was confirmed to be Tax by Western analysis using anti-tax antisera (section 2.2.3), (figure 3.7, panel C).

3.2.2.2 Characterisation of N-Tax

(a) The effect of N-Tax on CREB binding to HTLV-I and somatostatin promoters

Many reports in the literature have shown that Tax can increase the binding affinity of CREB for DNA. In addition, some groups have reported a small supershift of the CREB band indicative of a ternary complex. Therefore, although Tax does not bind to DNA directly, the gel mobility shift assay can be used to obtain functional information on the ability of Tax to interact with CREB. As an assay for the function of baculovirus N-Tax, increasing amounts of HSS1 and HSS2 N-Tax were titrated against set levels of CREB on the HTLV-I and somatostatin promoters. Titration of HSS1 N-Tax on the HTLV-I 147 bp probe resulted in an increase in binding affinity of CREB (figure 3.8, panel A). This increase in CREB affinity was specific to the HTLV-I LTR as titration of the same levels of N-Tax on the somatostatin probe had no effect of CREB affinity (figure 3.8, panel C). Interestingly, HSS2 N-Tax had no effect on the affinity of CREB for the HTLV-I LTR (figure 3.8, panel B). Therefore HSS1 N-Tax was used as the baculovirus N-Tax in all subsequent experiments. Although N-Tax altered the affinity of CREB for HTLV-I DNA there was no evidence of a supershift indicative of Tax/CREB complex formation on the probe.

(b) The effect of Tax on phosphorylated CREB binding to the HTLV-I LTR

Since the binding affinity of CREB is altered by phosphorylation, the effect of Tax on phosphorylated CREB was investigated and shown in figure 3.9. Titration of N-Tax on the HTLV-I LTR resulted in an increase in both CREB and P-CREB affinity (panel A, compare lanes 2-5 and 7-10 and panel B, compare 2-5 and 7-10). However, addition of Tax to the somatostatin 180 bp probe, even at high levels, had no effect on the affinity of either CREB or phosphorylated CREB (Panel C, compare lanes 2-5 and lanes 7-10).

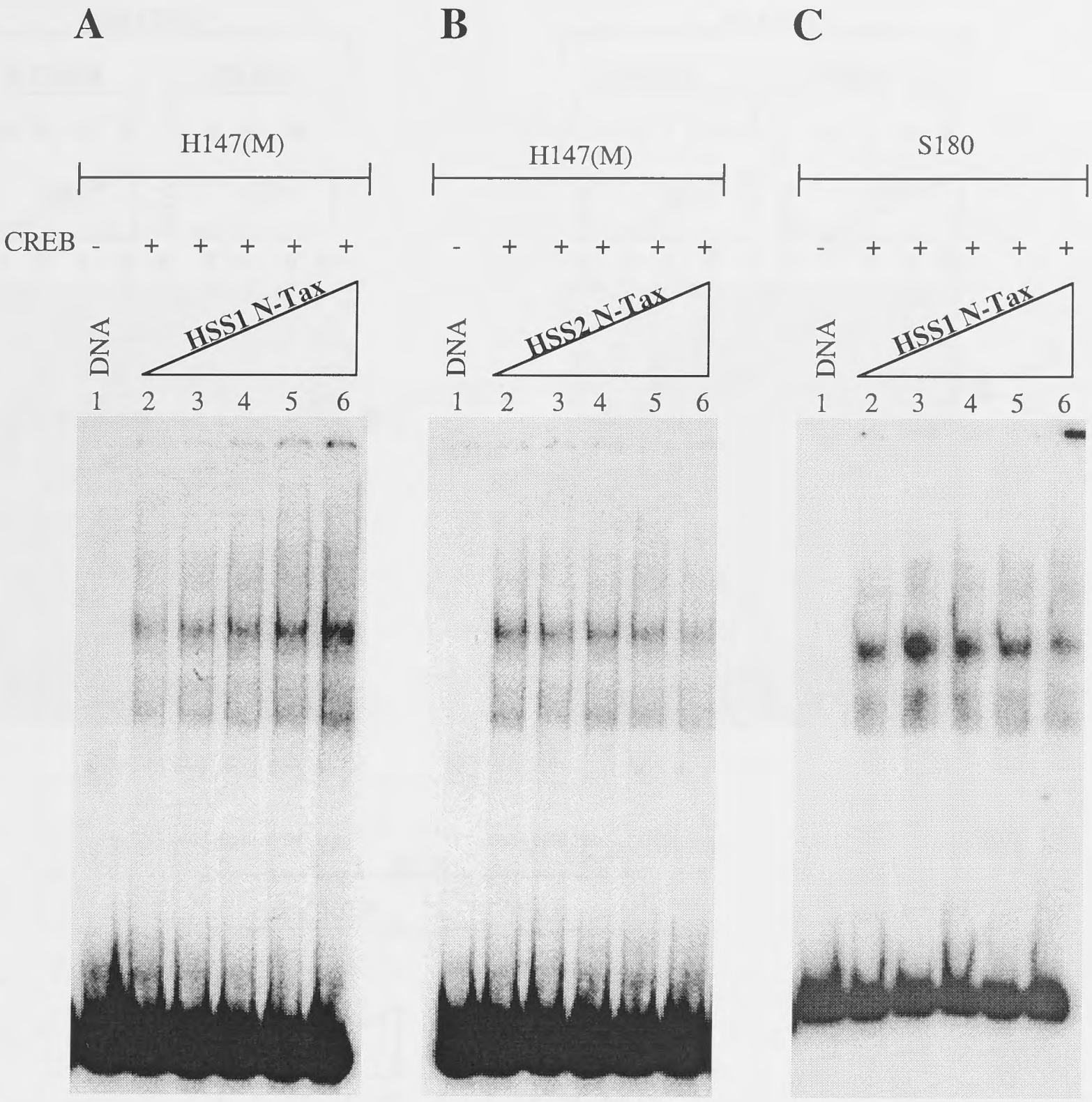


Figure 3.8: HSS1 N-Tax increases the affinity of CREB for the HTLV-I probe. Increasing concentrations N-Tax preparations were added to a constant concentration of CREB (2 μg), bound to the HTLV-I or somatostatin probe, and assayed by gel mobility shift. **A**. The effect of HSS1 N-Tax on CREB binding to the HTLV-I probe. Lane 1: Naked DNA; lanes 2-6; 2μg CREB and 0, 20, 100, 200 and 400 ng of HSS1 N-Tax respectively. **B**. The effect of HSS2 N-Tax on CREB bound to the HTLV-I probe. Lanes as for panel A. **C**. The effect of HSS1 N-Tax on CREB binding to the somatostatin probe. Lanes as for panel A.

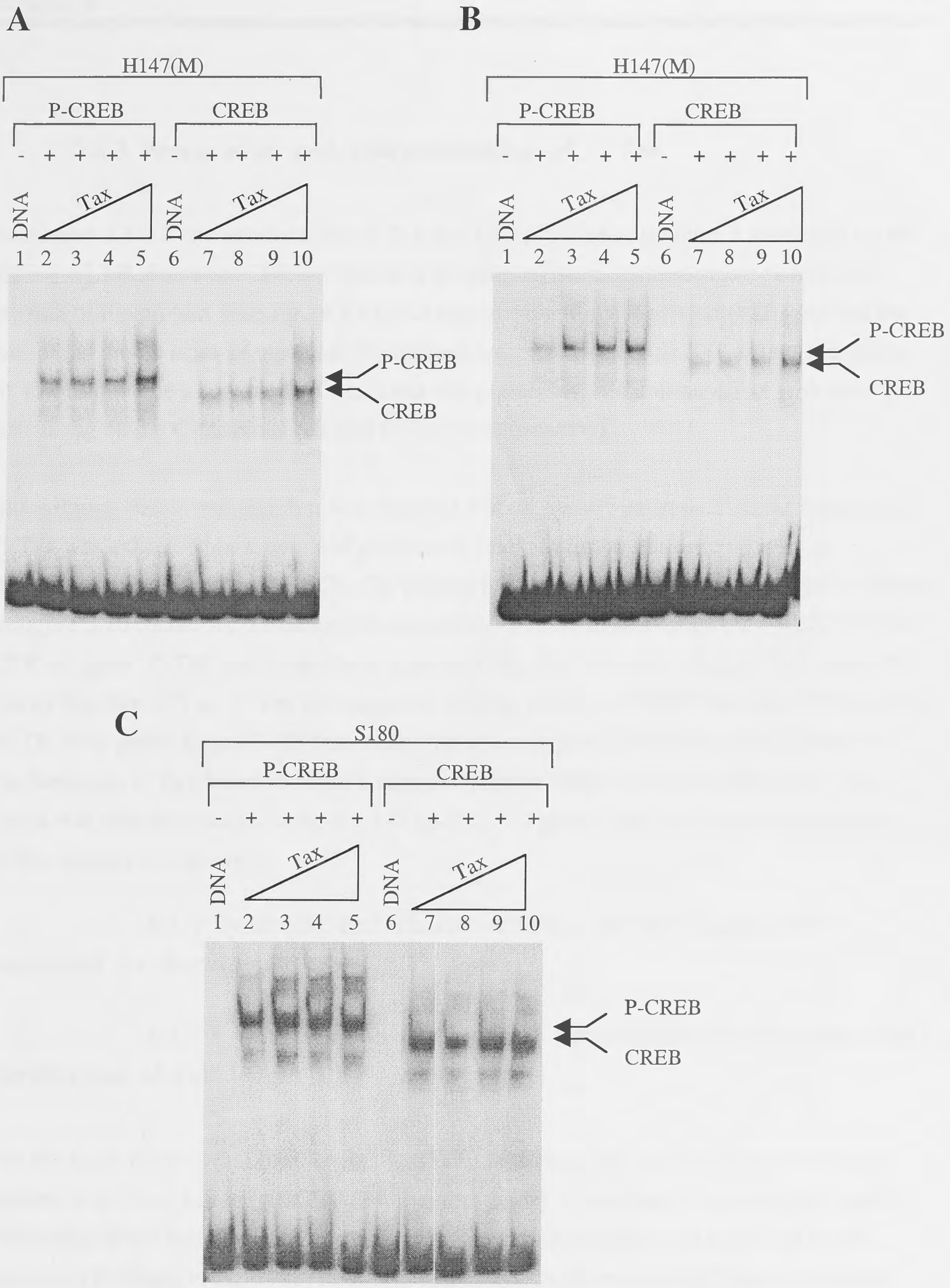


Figure 3.9: N-Tax increases the affinity of phosphorylated CREB for the HTLV-I probe. Increasing concentrations of HSS1 N-Tax were added to 2 μg of CREB or phosphorylated CREB (P-CREB) on HTLV-I or somatostatin probes and assayed by gel mobility shift. **A**. Effect of low concentrations of N-Tax on CREB binding to the HTLV-I probe. Lanes 1 and 6: Naked DNA; lanes 2-5 and lanes 7-10: 0, 20, 100 and 400 ng of Tax and 2 μg of either P-CREB (lanes 2-5) or CREB (lanes 7-10); **B**. The effect of higher concentrations of Tax on CREB binding to the HTLV-I probe. Lanes are as for panel A except Tax concentrations were 0, 250, 350 and 500 ng. **C**. The effect of N-Tax on CREB binding to the somatostatin probe. Lanes as for panel A.

3.2.3 Preparation and characterisation of C-Tax

In section 3.2.2.2, the combination of Tax and CREB failed to produce a supershift on the HTLV-I LTR. Since the CREB interaction domain on Tax is located on the N-terminal portion of the protein (see figure 1.9), it is possible that the N-terminal 6xHis tag and the additional amino acids of upstream N-terminal sequence (see figure 3.6) affects the ability of Tax to produce a supershift. To rule out this possibility, recombinant Tax with the 6XHis tag on the C-terminus (termed C-Tax) was generated.

Since during this investigation it was reported that bacterially expressed Tax is functional, C-Tax was expressed in *E.coli* and purified by Nickel affinity chromatography as described in the methods (2.3.1.2). The elution profile of C-Tax from the column is shown in figure 3.10 (panel A). To determine whether C-Tax could supershift the CREB HTLV-I LTR complex, C-Tax was titrated into a gel mobility shift reaction. Figure 3.10 (panel B) shows that like N-Tax, C-Tax increased the binding affinity of CREB for the HTLV-I LTR dLTR 30bp probe (lanes 7-10) but not for the somatostatin CRE 30 bp probe (lanes 2-5). Furthermore, C-Tax failed to cause a supershift of the CREB HTLV-I LTR band. This result was also reproduced using the 147 bp HTLV-I probe, and the 180 bp somatostatin probe (results not shown).

3.2.4 Synthesis and characterisation of full length CBP expressed by baculovirus

3.2.4.1 Construction of the CBP recombinant baculovirus and purification of full length CBP

For the same reasons that baculovirus was used to express N-Tax, this system was also chosen to produce full length CBP (2442 amino acids). In addition, the eukaryotic codon preference of the baculovirus system enables the accurate synthesis of large full length proteins (Williams *et al.*, 1988; Ikemura, 1984). cDNA for mouse CBP was sub-cloned into the FastBac transfer plasmid in frame with an N-terminal 6xHis tag (section 2.3.2b). A map of resulting transfer plasmid termed pCBP-Bac, is shown in figure 3.11 (panel A).

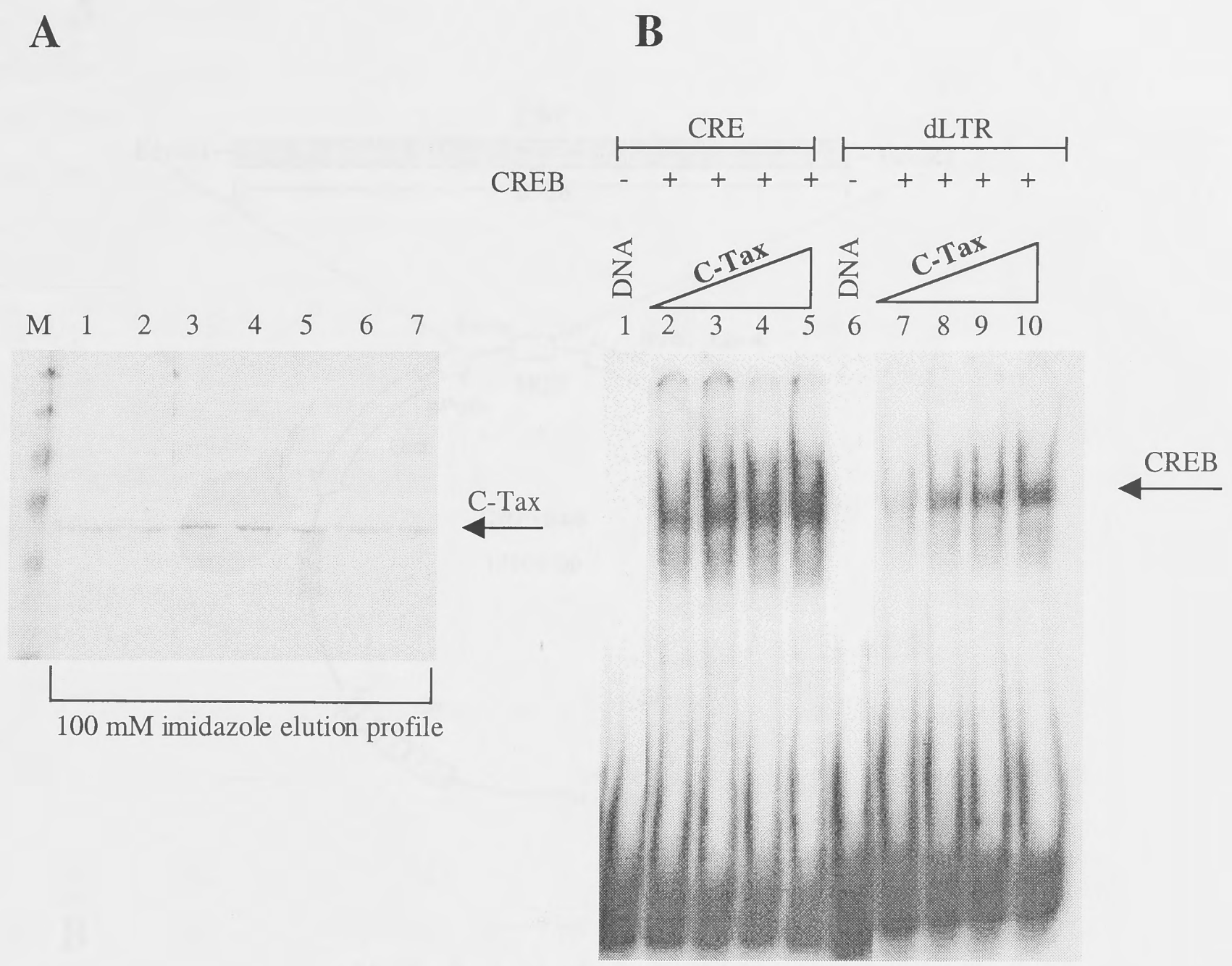
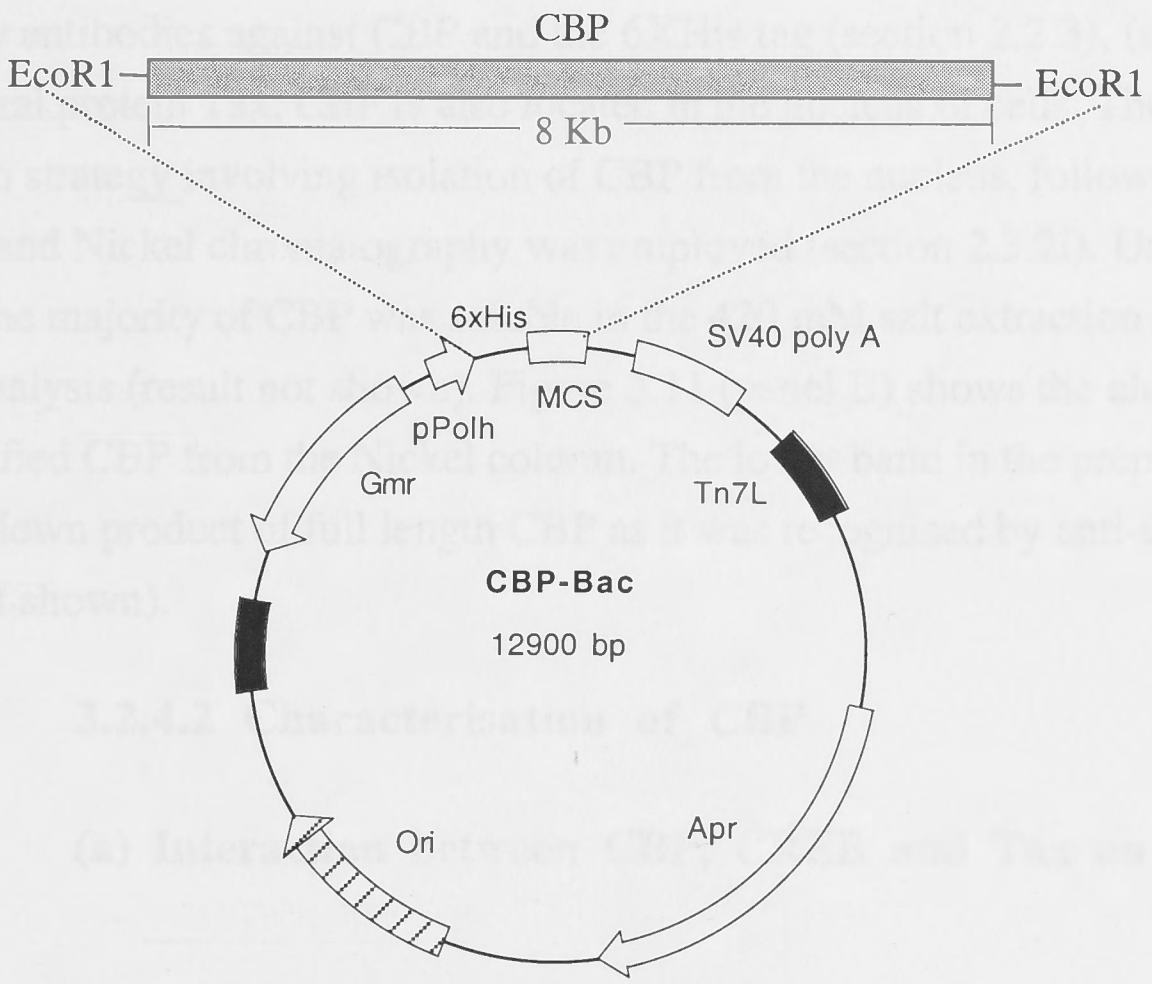


Figure 3.10: Purification of C-Tax and the effect of C-Tax on CREB binding to the HTLV-I or somatostatin probes. A. 100 mM imidazole elution profile of C-Tax from the nickel column run on 10% SDS-PAGE and visualised by Coomassie brilliant blue staining. **B**. Increasing concentrations of C-Tax were added to CREB and bound to the HTLV-I or somatostatin 30 bp probes. Lane 1: Naked DNA; lanes 2-5: 0, 1, 2 and 3 μg of Tax and 2 μg of CREB on the somatostatin CRE 30 bp probe; lane 6: Naked DNA; lanes 7-10: 0, 1, 2 and 3 μg Tax and 2 μg CREB bound to the HTLV-I dLTR 30 bp probe.

A



B

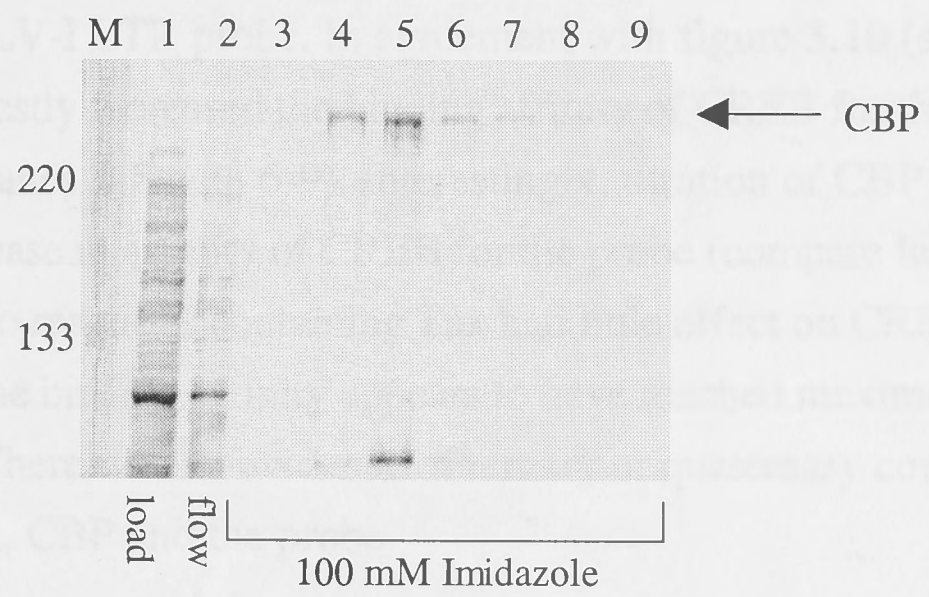


Figure 3.11: Sub-cloning and purification of full-length CBP. A. A schematic plasmid map of the pCBP-Bac transfer vector used to create the recombinant CBP baculovirus. **B**. Silver stained 7% SDS-PAGE of the CBP purification process. Lane 1: Salt extracted CBP preparation that was loaded on the column; Lane 2: Flow-through from the nickel column; lanes 3-9: 100 mM imidazole elution profile of CBP form the nickel column.

Recombinant virus was generated as described in the methods (section 2.3.2) and after infection with the recombinant bacmid, Sf9 cells expressed CBP as detected by Western analysis by antibodies against CBP and the 6XHis tag (section 2.2.3), (results not shown). Like the viral protein Tax, CBP is also located in the nucleus of cells. Therefore a similar purification strategy involving isolation of CBP from the nucleus, followed by salt extraction and Nickel chromatography was employed (section 2.3.2i). Unlike Tax however, the majority of CBP was soluble in the 420 mM salt extraction as detected by Western analysis (result not shown). Figure 3.11 (panel B) shows the elution profile of highly purified CBP from the Nickel column. The lower band in the preparation appears to be a breakdown product of full length CBP as it was recognised by anti-CBP antibodies (results not shown).

3.2.4.2 Characterisation of CBP

(a) Interaction between CBP, CREB and Tax on the HTLV-I LTR

To determine whether CREB, C-Tax and CBP form a quaternary complex with the HTLV-I LTR, C-Tax and CBP were titrated into mobility shift reactions containing fixed levels of CREB and 147 bp HTLV-I LTR probe. In agreement with figure 3.10 (section 3.2.3), addition of C-Tax modestly increased the binding affinity of CREB for the HTLV-I LTR (figure 3.12, compare lanes 2-5 with 6-9). Interestingly, titration of CBP into the reaction also resulted in an increase in affinity of CREB for the probe (compare lanes 3-5 with lane 2). Titration of CBP into reactions containing Tax had little effect on CREB affinity (compare lanes 7-9). The binding affinity appears to have reached maximum level after the first addition of CBP. There was no evidence of ternary or quaternary complex formation between CREB, C-Tax, CBP and the probe.

(b) Acetylation of core histones proteins by CBP

To further ascertain the function of recombinant full length CBP, the acetylation activity of the protein was investigated. In order to examine acetylation activity, a conventional HAT assay was employed. The HAT assay involves the transfer of ^{3}H-labelled acetate from acetyl-coA to the histone substrate. The labelled proteins are subsequently detected by visualisation of the protein by SDS-PAGE and flurography (section 2.10). First, the ability of CBP to acetylate the core histone proteins was examined. Figure 3.13 (lanes 7 and 8)

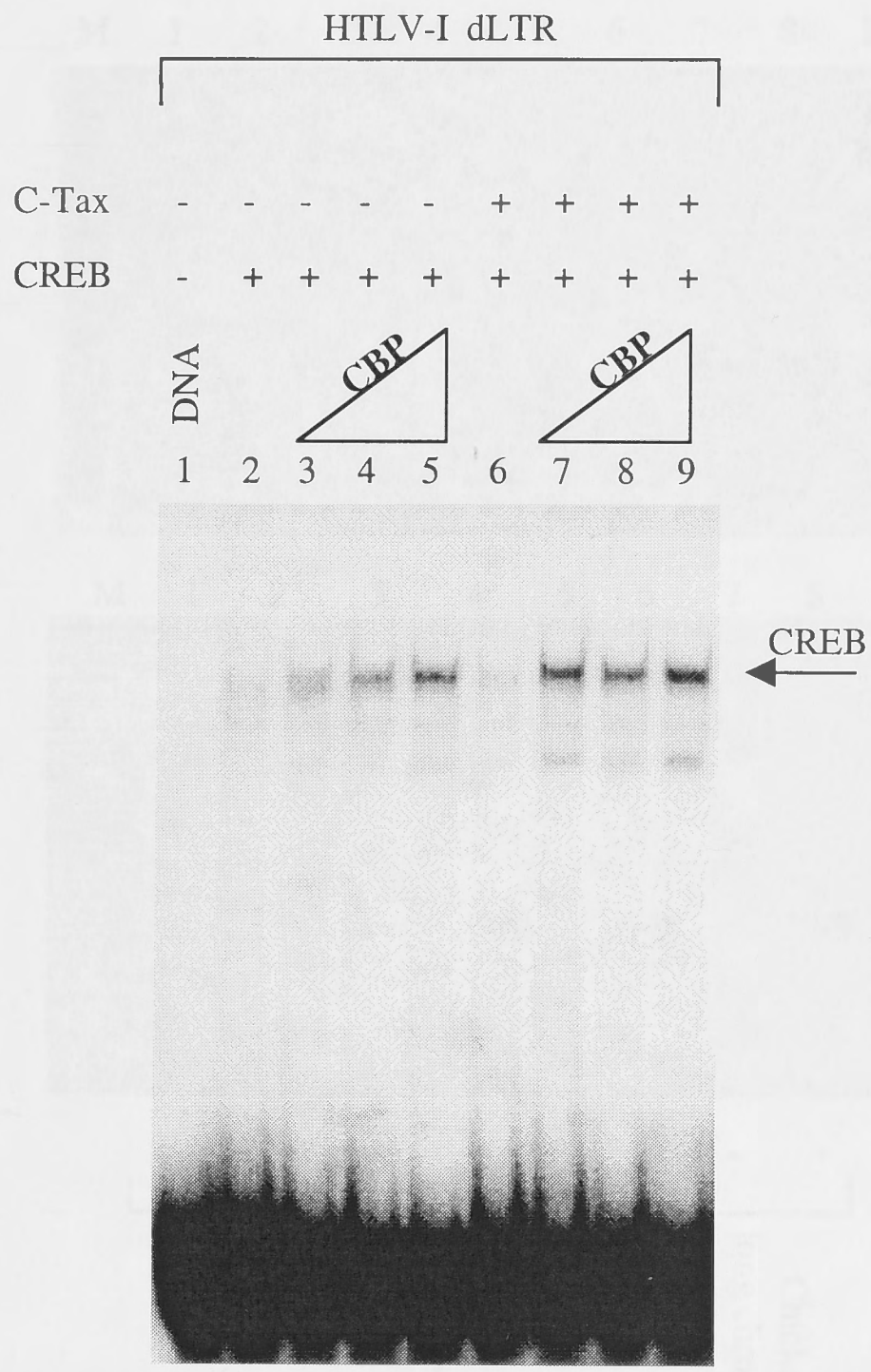


Figure 3.12: CBP increases the affinity of CREB for HTLV-I DNA. Increasing concentrations of CBP were titrated into constant concentrations of CREB or CREB and C-Tax on the 30 bp dLTR HTLV-I probe, and assayed by gel mobility shift. Lane 1: Naked DNA; lane 2-5: 2 μg of CREB and 0, 20, 40 and 60 ng of CBP respectively; lanes 6-9: 2 μg CREB, 40 ng C-Tax and 0, 20, 40 and 60 ng of CBP respectively. The position of the CREB/DNA complex is indicated by an arrow.

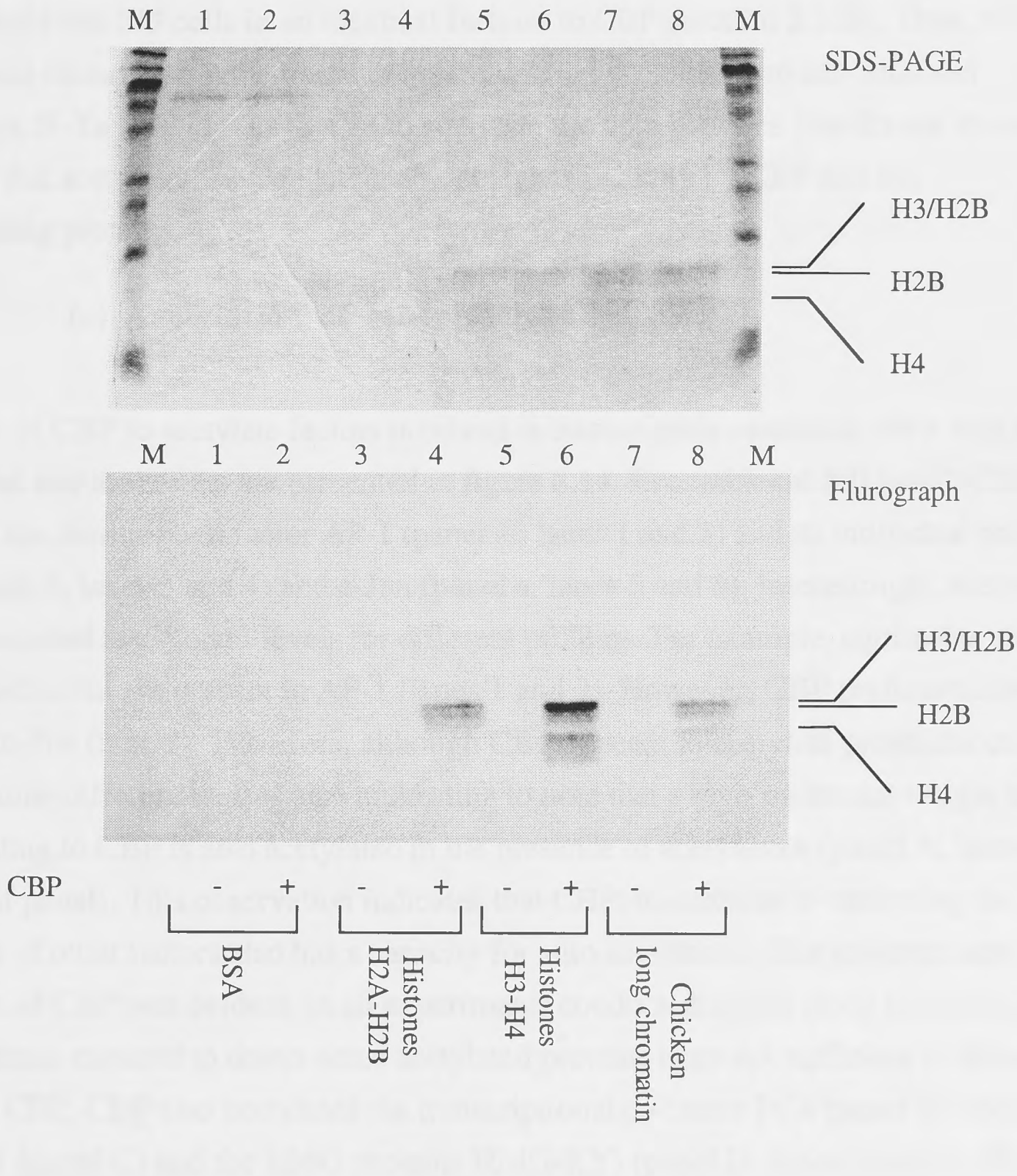


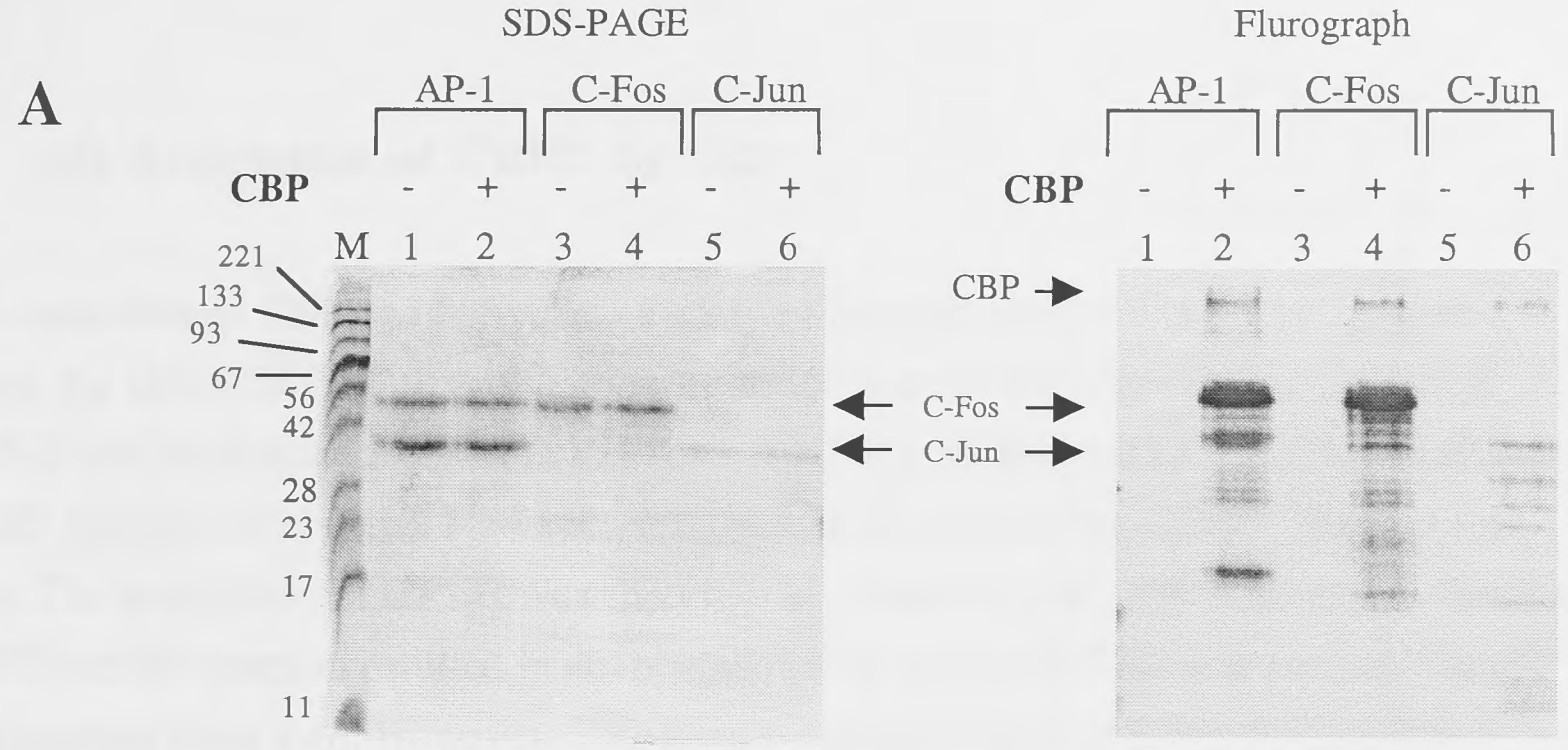
Figure 3.13: CBP acetylates the four core histones. 40 ng CBP was added to histones in the presence of H3-CoA, and analysed by HAT assay. Top panel is a Coomassie brilliant blue stained 18% SDS-PAGE and the bottom panel is the same gel visualised by flurography. CBP is present in the even numbered lanes only. Lanes 1 and 2: 2 μg BSA; lanes 3 and 4: 1 μg H2A/H2B dimer purified from long chicken chromatin (the concentration of H2A/2B is not sufficient for visualisation after treatment of the gel for flurography); lanes 5 and 6: 2 μg H4/H4 tetramer purified from long chicken chromatin; lanes 7 and 8: 1 μg of chicken long chromatin. Molecular weight standards (kDa) are shown at either side of the figure.

clearly shows that CBP is able to catalyse the acetylation of all four core histones in the nucleosome purified from long chicken chromatin (section 2.11.1.1). In addition, CBP also acetylates the purified H2A/H2B dimer (section 2.11.1.5) (Lanes 3 and 4) and the H3/H4 tetramer (section 2.11.1.6), (lanes 5 and 6). The acetylation activity of CBP is specific to the core histones as no acetylation of BSA was detected (lanes 1 and 2). As an additional control, N-Tax (HSS1) was used to acetylate the core histones. N-Tax (HSS1) was purified from Sf9 cells in an identical fashion to CBP (section 2.3.2i). Thus, N-Tax HSS1 would contain the same traces of contaminating proteins from Sf9 cells and baculovirus. N-Tax HSS1 was unable to acetylate the core histones (results not shown) indicating that acetylation is a direct result of catalytic activity by CBP and not contaminating proteins.

(c) Acetylation of other factors by CBP

The ability of CBP to acetylate factors involved in transcription control *in vitro* was also investigated and the results are presented in figure 3.14. Recombinant full length CBP acetylated the transcription factor AP-1 (panel A, lanes 1 and 2) and its individual proteins c-Fos (panel A, lanes 3 and 4) and c-Jun (panel a, lanes 5 and 6). Interestingly, acetylation by CBP occurred at different levels for different proteins. For example, equimolar amounts of c-Fos and c-Jun are present in AP-1 (lanes 1 and 2). However, CBP preferentially acetylates c-Fos (lane 2). Therefore, although CBP appears to acetylate promiscuously, there are some differences. It is also interesting to note that a high molecular weight band corresponding to CBP is also acetylated in the presence of acetyl-coA (panel A, lanes 2, 4 and 6, right panel). This observation indicates that CBP, in addition to catalysing the acetylation of other factors also has a capacity for auto acetylation. The apparent auto acetylation of CBP was evident in all experiments conducted in this study however, short exposure times required to detect some acetylated proteins were not sufficient to detect auto acetylated CBP. CBP also acetylated the transcriptional co-factor PC4 (panel B), the linker histone H1 (panel C) and the HMG proteins HMG-I(Y) (panel D, lanes 3 and 4), HMG 14 (panel D, lane 5) and HMG 1 (panel D, lanes 1 and 2). Following these experiments, the acetylation of HMG-I(Y), HMG 14 and H1 have since been reported in the literature (Herrera *et al.*, 1999; Munshi *et al.*, 1998; Bergel *et al.*, 2000)

Figure 3.14: CBP acetylates non-histone proteins. Left panel shows 10 % (panel A) or 15% (panels B, C, and D) SDS-PAGE stained with Coomassie brilliant blue before soaking in flurography reagent. The right panel shows a flurograph of the same SDS-PAGE. **A**. Acetylation of c-Fos, c-Jun and AP-1 by CBP. Even numbered lanes contain 40 ng of CBP; lanes 1 and 2: 5 μg AP-1; lanes 3 and 4: 5 μg c-Fos: Lanes 5 and 6: 1 μg c-Jun. **B**. Acetylation of PC4 by CBP. Lane 1: 5 μg PC4; lane 2: 5 μg PC4 and 40 ng CBP. **C**. Acetylation of histone H1 by CBP. Lane 1: 5 μg H1; lane 2: 5 μg H1 and 40 ng CBP. **D**. Acetylation of HMG proteins by CBP. Odd numbered lanes contain 40 ng CBP. Lanes 1 and 2: 5 μg HMG-1; lanes 3 and 4: 5 μg HMG-I(Y); lane 5 contains 2 μg HMG-14. The position of each protein is indicated by an arrow. Molecular weight standards (kDa) are shown at the left of each figure.


A
SDS-PAGE
Flurograph
AP-1
C-Fos
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- + - + - +
M 1 2 3 4 5 6
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HMG-1 HMG-I(Y) HMG-14
CBP + - + - + + - + - +
M 1 2 3 4 5 1 2 3 4 5
67
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23
17
11
CBP
HMG-1
HMG-14
HMG-I(Y)

(d) Acetylation of CREB by CBP

Since recombinant CBP can acetylate a variety of proteins involved in transcriptional control, the ability of CBP to acetylate proteins involved in the transcription control of HTLV-I was investigated (figure 3.15, panel A). The viral protein Tax, was not acetylated by CBP (results not shown). However, CBP was able to acetylate CREB, albeit at low levels. The acetylation of CREB was shown to be dependant on CBP however, as titration of CBP into the reaction resulted in an increase in the level of CREB acetylation. (figure 3.15 compare lanes 2-6). However, CREB was acetylated only at a low level as the strongest band on the gel is the auto acetylated CBP. The lower molecular weight acetylated bands on the flurograph are likely to be break-down products or contaminants from the CREB purification process as these bands become visable on SDS-gels which are stained with silver (results not shown). To determine if CREB phosphorylation had an effect on acetylation, phosphorylated CREB was acetylated by CBP. Figure 3.15 (panel B), shows no difference in the levels of acetylation between nonphosphorylated and phosphorylated CREB.

(e) Effect of acetylation on DNA binding

To determine whether acetylation affected the DNA binding ability of CREB, HMG-I(Y) and AP-1, gel mobility shift reactions were performed. The H147(M) probe was used in this study as it has been shown to bind CREB (section 3.2.1.2), AP-1 (section 1.8.1) and HMG-I(Y) (chapter 6). As the acetylation of the factors appears to be dose responsive (see figure 3.15), increasing amounts of CBP were used to acetylate CREB, HMG-I(Y) and AP-1 with unlabelled acetyl-coA as described in the methods (section 2.10) prior to their addition to the gel shift binding reaction. Although CBP was not removed from the DNA binding reaction, dilution of the acetylated protein into the binding reaction resulted in small amounts only of CBP present in the binding reaction. Figure 3.16 shows that acetylation of these factors, even with high levels of CBP had no effect on the binding affinities of these factors to the HTLV-I 147 bp probe.

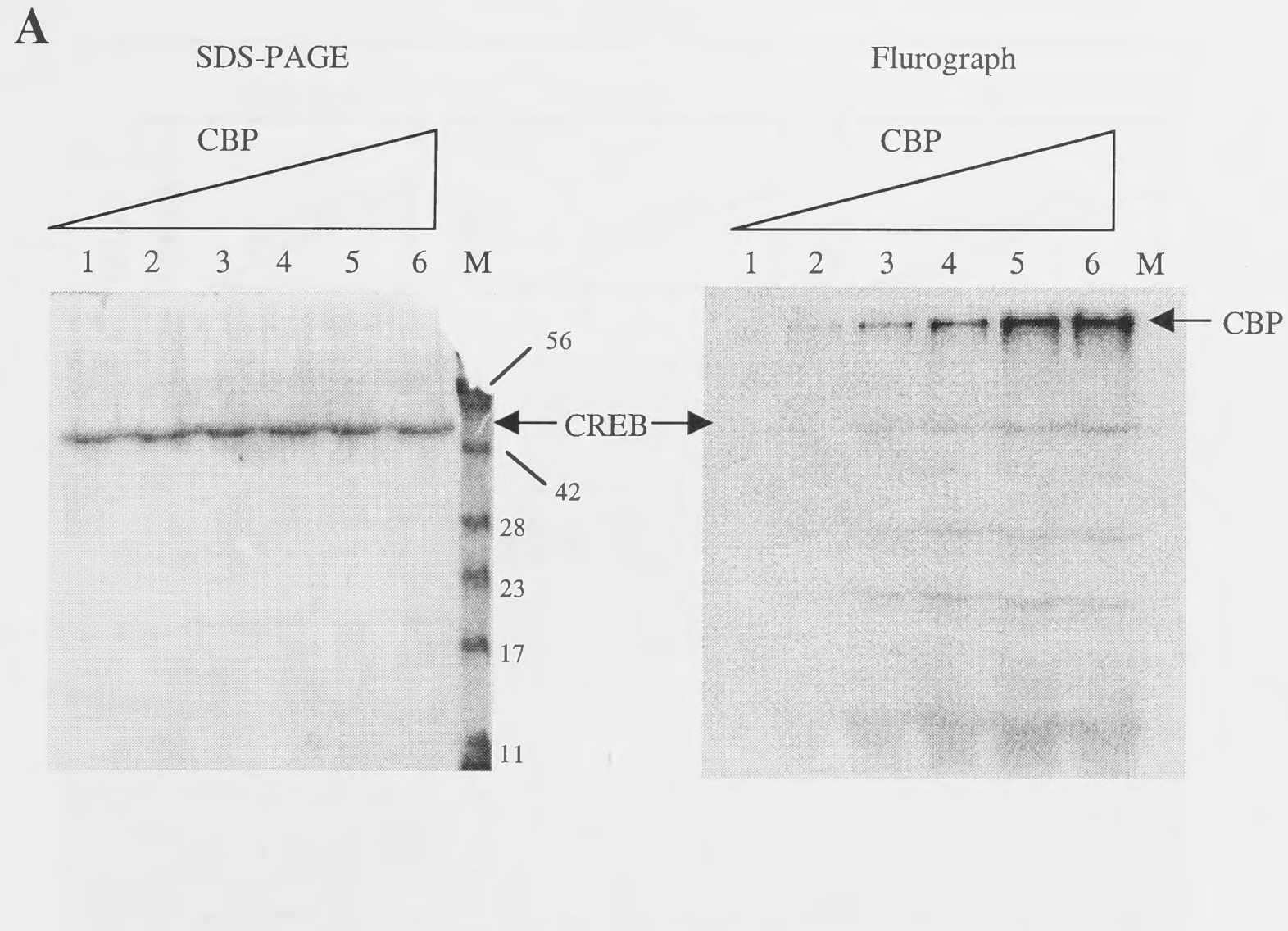


Figure 3.15 Acetylation of CREB by CBP. Coomassie stained 10% SDS-PAGE is shown on the left and a flurograph is shown on the right. **A**. Increasing concentrations of CBP were added to a constant concentration of CREB in the presence of H3-CoA and acetylation levels were monitored by the HAT assay. Lanes 1-6: 5 μg CREB and 0, 20, 40, 80, 160, 200 ng CBP respectively. Molecular weight markers (KDa) are shown on the right. **B.** 2 μg CREB (lane 1) and 2 μg P-CREB (lane 2) were combined with 40 ng of CBP and CoA and acetylation activity was monitored by HAT assay. Molecular weight markers (KDa) are shown on the left.

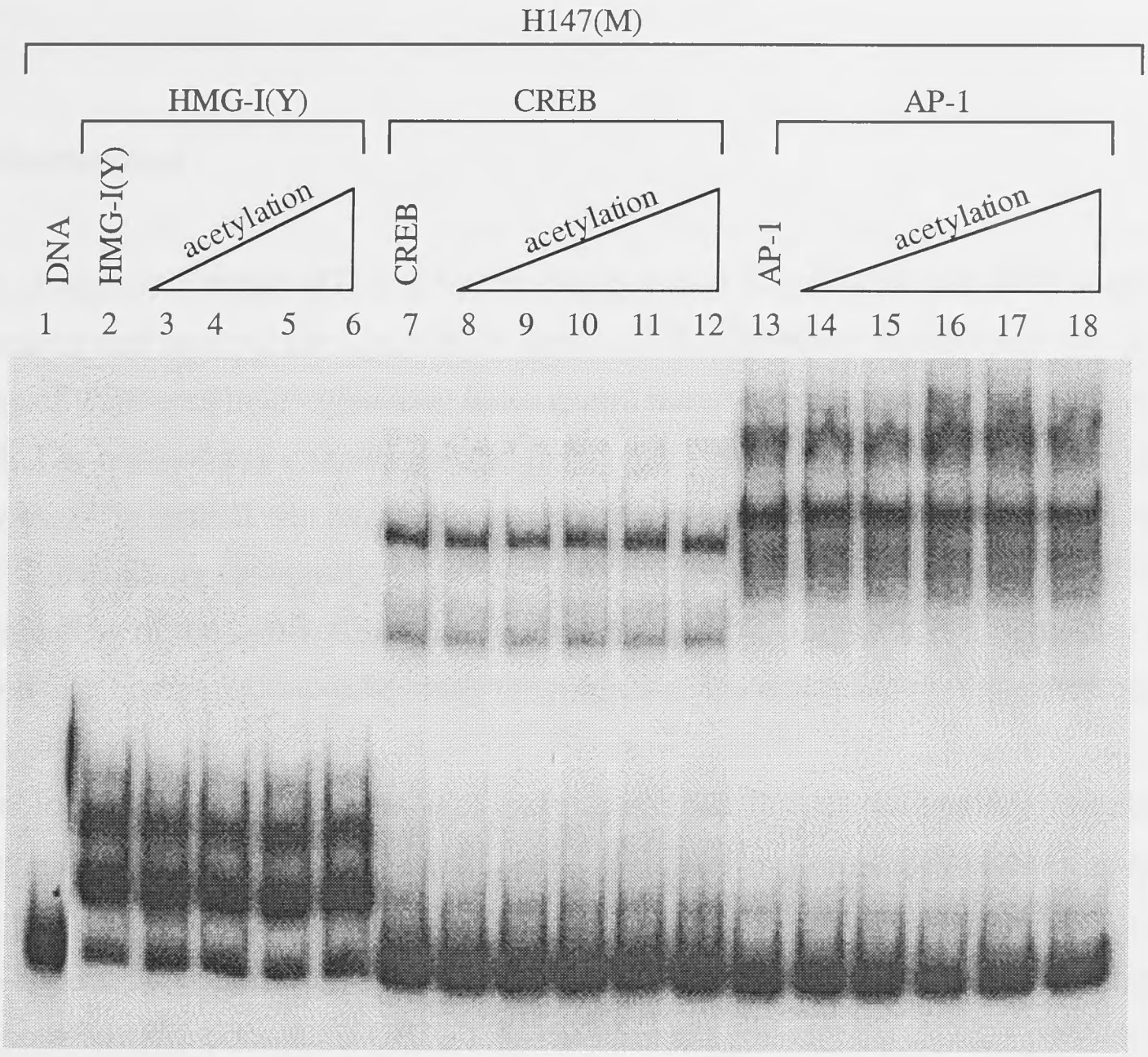


Figure 3.16 Acetylation of HMG-I(Y), CREB and AP-1 has no effect on DNA binding activity. 200 ng HMG-I(Y), 20 μg CREB and 50 μg of AP-1 were acetylated with unlabelled CoA by increasing concentrations of CBP. The effect of acetylation on binding to the HTLV-I LTR was monitored by a gel mobility shift assay using the H147(M) probe. Lane 1: Naked DNA; lanes 2-6 20 ng HMG-I(Y) acetylated with 0, 20, 40, 60, 80 ng CBP respectively; lanes 7-12: 2 μg CREB acetylated with 0, 20, 40, 60, 80 and 160 ng of CBP respectively; lanes 13-18: 5 μg AP-1 acetylated with 0, 20, 40, 60, 80 and 180 ng of CBP.

3.3 Discussion

In this chapter, the major HTLV-I interacting proteins Tax, CREB and CBP were synthesised and assayed for function in terms of DNA binding. In addition, the acetylation activity of CBP was investigated on histone and non-histone substrates including Tax and CREB. The aims of this chapter were to firstly show that the recombinant proteins were functional. The second aim involved characterising the interactions between the major HTLV-I interacting proteins both individually, and in combination, with naked DNA. An understanding of the interaction of these proteins with naked DNA is a necessary first step required as a comparison to determine the impact of nucleosomes on transcriptional control.

Recombinant CREB was expressed in bacteria and purified by the method commonly used my many investigators. In addition, a second purification step was developed to generate highly purified protein. Purified CREB was able to bind specifically to the TRE element of the HTLV-I LTR contained within an 147 bp DNA fragment, but at a lower affinity to that of the somatostatin consensus CRE. This result is in agreement with data published by other investigators (Kwok *et al.*, 1996).

The baculovirus expression system was chosen to express Tax and CBP to take advantage of the systems ability to post-translationally modify proteins. In addition, codon preferences in the baculovirus system compared to eukaryotic systems, enable the translation of large proteins such as CBP (Williams *et al.*, 1988; Ikemura, 1984). The bacmid system of creating the recombinant virus was employed, where cDNA for the required protein is transposed into the virus which exists as a bacmid in bacterial cells (methods 2.3.2). A drawback of using this system is the presence of empty bacmids in colonies that also contain recombinant bacmid. The presence of empty bacmid is undesirable as this DNA would be co-purified with the recombinant bacmid DNA and used to transfect Sf9 cells. It is reasonable to assume that there are negative selection pressures on the propagation of viruses which contain DNA superfluous to the survival of the virus. Thus, in a preparation of cells infected with both protein expressing virus and native virus, the native virus would have a selection advantage and eventually dominate the species of virus present in the preparation. Empty bacmid would limit the amount of recombinant protein found in these cells. In order to overcome this limitation, we showed that replating

the colonies onto fresh selection plates prior to isolation of DNA results in removal of empty bacmid. This step is vital to obtain high level expression of recombinant protein.

The insolubility of the Tax protein necessitated the development of a method to solubilise the protein for purification, while retaining full function of the protein. Insolubility of Tax has been noted by other laboratories including Mathews *et al.* (1992) and Jeang *et al.* (1997). It is interesting to speculate about the reasons why HSS1 N-Tax extracted with 420 mM salt was active, whereas HSS2 Tax, extracted with 840 mM salt, was inactive in gel mobility shift reactions. Mathews *et al.* (1992) also used salt extraction to solubilise Tax, and found that Tax extracted with 1M NaCl was inactive in *in vitro* transcription reactions. These workers subjected this preparation to gel filtration chromatography to separate monomers from aggregates and found that only monomeric Tax was active. Perhaps the Tax extracted in 840 mM NaCl is also largely present as an aggregate explaining why the HSS2 was not effective in gel shift reactions.

Although N-Tax increased the affinity of CREB for the HTLV-I LTR, N-Tax did not produce a supershift as has been demonstrated by other workers. One possible explanation for this is that the N-terminal 6XHis tag and other N-terminal non-coding sequence may inhibit the interaction between Tax and CREB as the CREB interaction domain of Tax is located at the amino terminus (Goren *et al.*, 1995; Yin *et al.*, 1995a). This possibility was tested by the use of recombinant Tax synthesised with a C-terminal 6XHis tag, expressed in bacteria. C-Tax behaves in an identical way in gel shift reactions and therefore the absence of a supershift may not be related to the presence of the extra N-terminal sequence.

Early reports in the literature also failed to detect the presence of a supershift between CREB and Tax (Yin and Gaynor, 1996a and b; Yin *et al.*, 1995 a and b; Armstrong *et al.*, 1993; Franklin *et al.*, 1993). One possible reason for the absence of a supershift is the electrophoresis conditions used. Generally, investigators that show a supershift use mild electrophoresis conditions such as low concentrations of salt in the gel and running buffer (0.25% TBE). In addition, the gels are pre-run and/or run for long periods of time to de-ionise the buffer conditions and more easily resolve the ternary complex. Indeed some groups have proposed that a very slight, barely detectable supershift is present (Zhao and Giam 1992; Suzuki *et al.*, 1993; Anderson and Dynan, 1994). Another common element in groups that demonstrate definite supershifts is the use of truncated CREB such as CREB-BR or chimeric forms of CREB (Adya *et al.*, 1994; Brauweiler *et al.*, 1995). Indeed, it has been demonstrated that the interaction between Tax and truncated CREB is considerably

stronger than the interaction between Tax and full length CREB (Brauweiler *et al.,* 1995). The best explanation to accommodate these seemingly conflicting results is that Tax and CREB form a weak association with a rapid equilibrium between the associated and disassociated states. The ternary complex may dissociate in the mobility shift reaction depending on the electrophoresis conditions used. Evidence for this proposal comes from the detection of ternary complexes by other methods besides gel mobility shift such as supershift analysis with Tax antibody (Zhao and Giam, 1992; Paca-Uccaralertkun *et al.*, 1994), immobilised templates (Yin and Gaynor, 1996a) and the mammalian two-hybrid system (Yin and Gaynor 1996b). Interestingly Yin and Gaynor (1996a and b) were unable to detect a supershift in gel mobility shift reactions. The results from this chapter and others show a Tax mediated increase in affinity of CREB for the HTLV-I LTR and not the somatostatin CRE. To achieve an increase in binding on the HTLV-I LTR only, Tax and CREB must at least transiently interact on the HTLV-I LTR. Therefore, recombinantly expressed Tax by both bacterial and baculoviral methodologies retains the function ascribed to the native Tax protein *in vivo*.

In this study and others (for example Kwok *et al.*, 1996), it was shown that the binding affinity of CREB for the consensus CRE element found in the somatostatin promoter is of a higher affinity than binding to the HTLV-I TRE. The reasons for the differential affinity may be the small difference in the core sequence (figure 1.8b). In the HTLV-I LTR the three 21-bp repeats also differ slightly in core sequence (Figure 1.8b). Therefore it is interesting to determine whether these differences in core sequence can affect the affinity of CREB in a similar way to the somatostatin promoter. Several investigators have compared the relative CREB binding affinities of the TREs and demonstrated differences in the binding affinities of CREB (Brauweiler *et al.,* 1995; Yin *et al.,* 1995a). However other laboratories have not shown any difference in CREB binding affinity (Goren *et al* 1999; Anderson and Dynan 1994). The reasons for the differences in these results are unclear. In this chapter it was demonstrated that the affinities of the TREs differed with the distal LTR having the highest affinity for CREB where as the proximal LTR having the lowest (figure 3.4). This hierarchy of CREB affinity is in complete agreement with the hierarchy shown by Yin *et al.* (1995a). Published studies have shown that mutation of any one of the three TREs affects transcription differentially (Goren *et al* 1999). Different affinities of CREB to each of the three LTRs may explain this observation.

In addition to the binding affinity of CREB being determined by the core sequence, this study has shown that phosphorylation of CREB can also increase the proteins binding

affinity. In addition, the overall affinity of phosphorylated CREB for the HTLV-I LTR is greater in combination with Tax, although the affinity of phosphorylated and nonphosphorylated CREB were similarly increased by Tax (figure 3.9). It is interesting to speculate on the mechanism by which phosphorylation can increase the binding affinity of CREB. CREB is phosphorylated at serine 133 *in vitro* and *in vivo* by PKA (Montminy, 1997). Although the phosphorylation site is unlikely to contact the DNA binding site directly (figure 1.11), phosphorylation may result in a conformational change which enables a higher level of binding. This suggestion is supported by the altered mobility of phosphorylated CREB in mobility shift reactions (figure 3.5).

In the literature there is disagreement over the extent to which phosphorylation of CREB influences DNA binding. For example Hagiwara *et al.* (1993) present evidence showing that phosphorylation of CREB has no affect on CREB affinity for the somatostatin CRE. However, work by Nichols *et al.* (1992) shows that phosphorylation has a profound effect on the binding of CREB to the low affinity TAT promoter and a mild increase in affinity on the somatostatin CRE. Furthermore Yin and Gaynor (1996b) show increased binding of phosphorylated CREB compared to unphosphorylated CREB in immobilised template assays. The demonstration in this chapter that phosphorylation increases the binding affinity of CREB on the lower affinity HTLV-I LTR correlate well with Nicholas *et al.* 1992 and Yin and Gaynor (1996b). However, work by Anderson and Dynan, (1994) on the HTLV-I promoter failed to show any difference in affinity between phosphorylated and nonphosphorylated CREB. The reason for these different results are unclear.

Many groups have reported an increase in HTLV-I transcription in response to stimulation of the phosphorylation signalling cascades in tissue culture cells (Saggioro *et al.*, 1994; Kadison *et al.*, 1990; Tan *et al.*, 1989; Copeland *et al.*, 1994; Poteat *et al.*, 1989, 1990). A major function of CREB phosphorylation is thought to be the recruitment of CBP (Shih *et al.*, 1996; Parker *et al.*, 1996). Recent solution of the phosphorylated CREB-CBP complex by nuclear magnetic resonance spectroscopy demonstrates the importance of phosphorylation for complex formation (Radhakrishnan *et al.*, 1997). However, the results in this chapter show that phosphorylation of CREB also results in an increase in the DNA binding affinity of CREB. In addition, this affinity is even greater in combination with Tax and like unphosphorylated CREB, the increased affinity of phosphorylated CREB in response to Tax was not evident on the somatostatin CRE. These results suggest that increased affinity or stabilisation of the CREB/Tax complex by phosphorylation may be part of the mechanism involved in the activation of viral transcription.

In this chapter full length CBP was successfully synthesised in the baculovirus system and purified by Nickel chromatography. It is important to note that the production of full length CBP had not been reported in the literature at this time. As a test of functionality of recombinant CBP, this study demonstrated that CBP was able acetylate the four core histones. This result is in agreement with Ogryzko *et al.* (1996) and Bannister and Kouzarides, (1996). The demonstration of functional acetylation activity indicates that the recombinant full length CBP retains at least some of the function of the *in vivo* protein.

It is well established that Tax, CREB and CBP can form a quaternary complex on the HTLV-I promoter. Evidence for a quaternary complex comes largely from fluorescence polarisation binding assays (Kashanchi *et al.*, 1998; Kwok *et al.*, 1996). Furthermore, similar to the Tax induced supershift of CREB binding to the HTLV-I LTR detected by some investigators, a truncated form of CBP known as GST-KIX has been shown to form a quaternary complex with Tax and CREB on the viral LTR (Giebler *et al.*, 1997; Lenzmeier *et al.*, 1998). In this chapter a quaternary complex was not detected between Tax, CREB and full length CBP. Indeed, if CBP had bound, the complex would be in the well. However, CBP did appear to increase the affinity of CREB for the HTLV-I LTR in combination with Tax. This result is consistent with Giebler *et al.*, (1997), who also demonstrated an increase in the affinity of CREB as a consequence of GST-KIX binding. One possible reason that a quaternary complex was not detected may be related to the use of full length CBP in this study. Truncated CBP may have a higher affinity for complex formation similar to truncated CREB (Brauweiler *et al.,* 1995). Evidence that full length CBP has different functions is supported by transcription experiments that show that truncated CBP can activate transcription more efficiently the full length CBP (Swope *et al.*, 1996). However the possibility that the absence of a quaternary complex involves experimental techniques such as differences in electrophoresis conditions cannot be discounted. The observation that CBP, in combination with Tax, can increase the affinity of CREB for the HTLV-I promoter indicates that there must be a transient interaction between these proteins and the HTLV-I LTR and supports the notion that CREB, Tax and CBP can interact on the HTLV-I promoter.

This study has demonstrated that CBP, in addition to histone acetyltransferase activity, is able to acetylate other factors involved in transcription *in vitro*. Factors acetylated by CBP in this study include CREB, PC4 (a transcriptional co-activator), histone H1, c-Fos, c-Jun and the HMG proteins 1, 14 and I(Y). Although CBP seems to acetylate non-

discriminately, there does appear to be some differences in acetylation efficiency across different proteins. Since the demonstration of this ability, other investigators have also reported the acetylation of other factors by CBP. Of these factors, the best characterised include HMG-I(Y), GATA and p53. In addition to *in vitro* acetylation, the acetylation of these factors has also been demonstrated *in vivo* (Hung *et al.*, 1999; Munshi *et al.*, 1998; Liu *et al.*, 1999).

The precise function of the acetylation of factors is currently the subject of intensive investigation as it appears that the acetylation of factors does not have a global function. Acetylation of HMG-I(Y) at the β-INF enhanceosome results in decreased binding affinity and disruption of the enhanceosome (Munshi *et al.*, 1998). Furthermore, these investigators have shown that acetylation of HMG-I(Y) by a different acetylase, p/CAF, has no affect on DNA binding. In this study, acetylation of HMG-I(Y) by CBP has no effect on its ability to bind the HTLV-I promoter (figure 3.16). This observation is important because it indicates that the acetylation of the same protein by the same acetylase may have different functions depending on the gene context. By contrast, acetylation of p53 by CBP results in an increased affinity of p53 for DNA (Gu and Roeder, 1997). Similarly, the acetylation of the GATA 1 factor has been reported to result in an increased affinity for DNA (Boyes *et al.*, 1998). However work by Hung *et al.* (1999) shows no effect on GATA 1 DNA binding resulting from acetylation. These different findings have been attributed to the different species origin of the protein (chicken and mouse respectively) or between the enzymes used to acetylate them (CBP and p/300 respectively). Recently, Suzuki *et al.* (2000) identified the acetylation of SP1 by p300 and showed that although interaction with p300 resulted in stimulation of SP1 binding to DNA, this stimulation was not due to acetylation. From the investigations to date, it appears that *in vivo* function of factor acetylation varies considerably depending on the nature of the factor acetylated, the gene context, and the acetylating enzyme.

The functional relevance of acetylation of other protein factors in this study may be disputed as the acetylation of these factors has not been observed *in vivo*. However, the detection of the *in vivo* acetylation of these factors may be complicated if only a small portion of the protein is acetylated. For example, in this study and others (Herrera *et al.*, 1997), H1 is acetylated by CBP *in vitro* however, the acetylation of H1 has not been detected *in vivo*. Recent studies by Chen and Grovosky (1996) have shown that rather than having a global effect on gene expression by repression, H1 is involved in the expression of specific genes. Since H1 is involved in gene specific regulation and not global

transcriptional control then H1 may only be acetylated at specific target genes and not globally making detection of acetylation difficult. Furthermore, the transcriptional cofactor PC4 has been identified as a substrate for acetylation in this study, however PC4 cannot be acetylated while it is phosphorylated (Dr A. Halloway, personal communication). The majority of cellular PC4 is present in the phosphorylated state (Ge and Roeder, 1994), therefore detection of acetylation would be difficult even with the use of deacetylase inhibitors such as sodium butyrate and trichostatin. Nevertheless, it would be interesting to ascertain the acetylation states of these factors *in vivo*.

This study also shows that acetylation does not alter the binding activities of AP-1 and CREB on the HTLV-I LTR. However, because the acetylation may effect proteins differently, and the function of acetylation may also depend on the gene context, a functional role for the acetylation of these proteins cannot be ruled out. In addition, acetylation may not only involve protein-DNA interactions but may also involve protein-protein interactions. Evidence in support of the involvement of acetylation in protein-protein interactions comes from studies of histone acetylation. For example, it has been demonstrated that acetylation of histone tails H3 and H4 enable the tails to interact with the bromodomain of CBP, TAFII250 and possibly other proteins (Dhalluin *et al.*, 1999; Jacobson *et al.*, 2000).

In this study, CBP was shown to acetylate CREB in a dose dependant fashion. However, there have been no reports of the acetylation of CREB in the literature. One reason why CREB acetylation may not have been reported is that many investigators use the acetylation domain of CBP only, whereas in this work, full length CBP was used. The CREB interaction domain and the HAT domain of CBP are located at different positions of the protein (figure 1.7). Perhaps CREB is required to bind CBP before CBP can catalyse the acetylation reaction. If this were the case than phosphorylated CREB, which has been shown to bind CBP with a higher affinity than unphosphorylated CREB (Parker *et al.*, 1996), should be acetylated to a greater degree. However, in this study there is no apparent difference between the acetylation of phosphorylated and unphosphorylated CREB. Furthermore, as Tax has been shown to act as a bridging protein between CBP and CREB, the presence of Tax in the acetylation reaction may increase the acetylation of CBP. The addition of Tax in the acetylation reaction has no effect on the level of CREB acetylation (data not shown).

CREB was acetylated at a low level by CBP indicating that CREB has the capacity to be acetylated. However, CREB may not be the target of CBP acetylation *in vivo*, rather CREB may be the target of other enzymes that possess acetylation activity such as p/CAF and TFII50. In addition, acetylation of CREB had no affect on the DNA binding affinity of the protein. However, one possible way that acetylation of CREB could have a functional role is by protein/protein interactions between CREB and other transcription factors or co-factors.

By analysis of the known substrates for CBP acetylation a putative motif for acetylation can be defined (see figure 3.17). Interestingly CREB contains a similar motif. It would be interesting to determine the exact amino acid(s) of CREB that are acetylated by CBP. One way this could be achieved is by sequencing acetylated CREB. It would also be desirable to design peptides spanning the lysine residues of CREB. Firstly in order to rule out the possibility that a contaminating protein in the CREB preparation is responsible for the acetylation. Secondly, narrowing down the acetylation site using peptides would assist in N-terminal sequencing. In preliminary investigations in this study, the acetylation of CREB was further defined by determining the acetylated amino acids. Acetylated CREB was cleaved with p-Lys-S, an enzyme which cleaves proteins at every lysine residue. The protein fragments were separated using the SMART HPLC system (in collaboration with Dr P.J. Milburn) and counted to determine the fractions which contained the labelled peptides was counted. The fractions corresponding to the peak counts were N-terminally sequenced and found to correspond to two peptides from the C-terminus of CREB that contained the KALK motif (see figure 3.17). Although preliminary, this result indicates that either that lysine 330 and/or 333 can become acetylated on CREB.

This chapter has presented the synthesis and characterisation of the recombinant proteins CREB, Tax and CBP used throughout this study. The recombinant proteins show many of the functional characteristics attributed to the *in vivo* proteins and is consistent with the proposed model of interaction between Tax, CREB and CBP and the natural HTLV-I LTR promoter (see Figure 1.12). Importantly this study has extended these observations and has shown that modifications such as acetylation and phosphorylation may influence the control of HTLV-I transcription in subtle and specific ways as tested on naked DNA templates. However *in vivo*, the retrovirus LTR exists within the confines of chromatin environment. The chromatin environment not only represses the transcription of the virus but is a regulatory element involved in the control of transcription. Therefore the regulation of

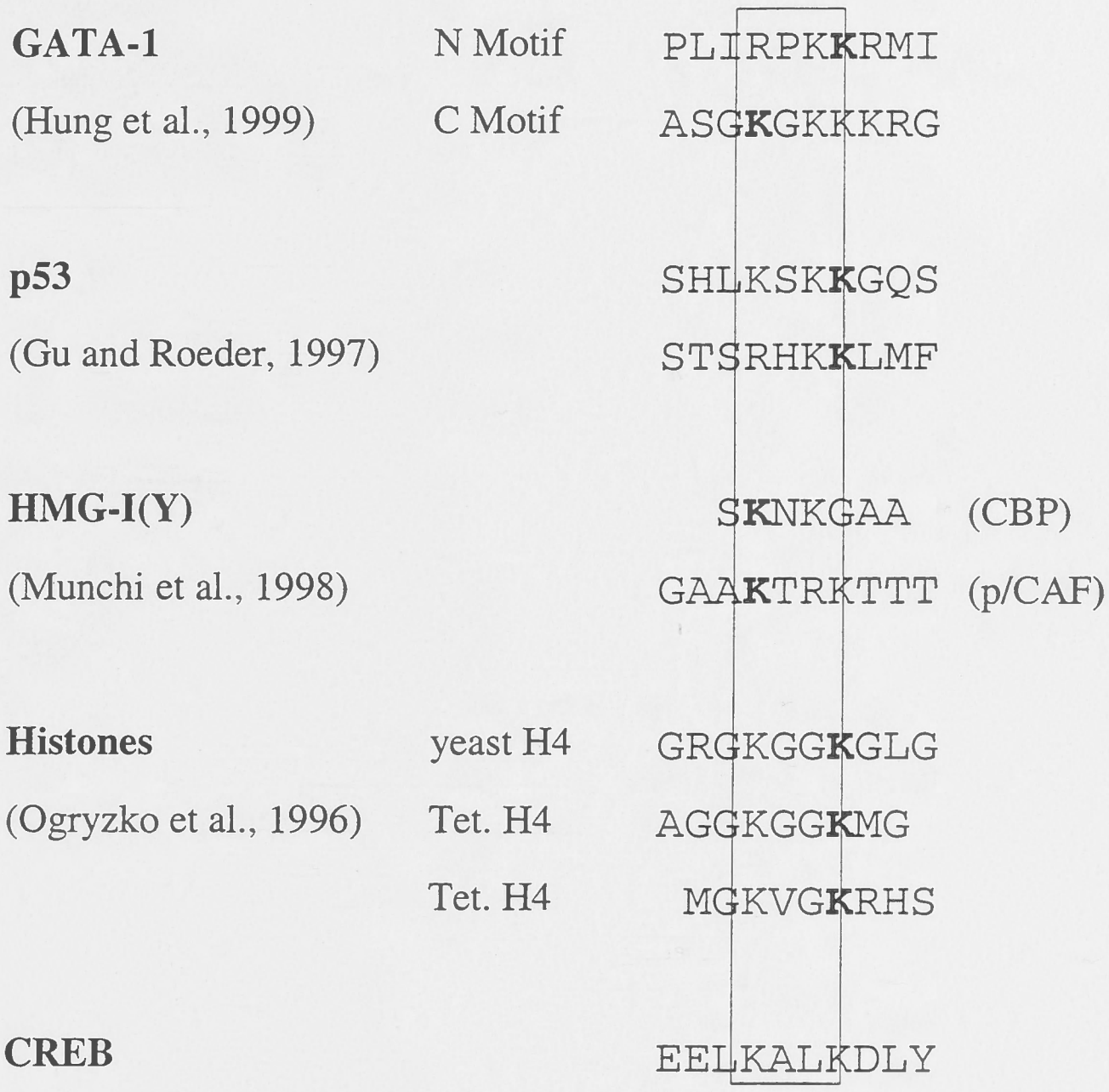


Figure 3.17: Sequence alignment of the acetylation sites of various proteins. The preferentially acetylated lysine is shown in bold.

HTLV-I transcription must take into account the high level of control exerted by nucleosomes.

CHAPTER 4:

The interaction of CREB with DNA assembled into a single nucleosome.

4.1 Introduction

Transcriptional regulation from an inducible gene generally involves the binding of one or several *trans*-active factors to their cognate DNA-binding sites within the promoter. DNA *in vivo* is complexed with histones to form nucleosomes and higher order chromatin structures. Therefore, nucleosome binding by sequence specific transcription factors may represent the initial step in the remodelling process that leads to inducible transcription *in vivo* (Li and Wrange *et al.*, 1993). The second step may be the recruitment of a remodelling complex or HAT activity which leads to the alteration of chromatin structure. Therefore, in order to investigate transcription of HTLV-I within chromatin, it is necessary to firstly characterise transcription factor nucleosome interactions on the HTLV-I LTR.

As just stated, the binding of a factor to a nucleosomal template may be an important initial step in the activation process. A principle observation in relation to transcription factor nucleosome interactions is that nucleosomes inhibit the association of regulatory factors with their cognate binding sites to differing degrees. At least four mechanisms govern the ability of factors to access their binding sites on nucleosomes.

a). A number of studies have suggested that several transcription factors may have inherent nucleosome binding abilities. For example, a GAL4-VP16 dimer binds to a single site in chromatin with a 100-fold decrease in affinity for a single site (relative to naked DNA) and only a 10-fold decrease in affinity for five consecutive sites (Taylor *et al.*, 1991). By contrast, studies of the human heat shock factor (HSF) have demonstrated that this factor is unable to bind to its sequence element regardless of rotational orientation or the number of binding sites (greater than 1000 fold increase in binding affinity), (Taylor *et al.*, 1991).

b). A second mechanism used in nucleosome DNA interactions is synergism between different factors. For example, on the Mouse Mammary Tumour Virus (MMTV) promoter, binding of the glucocorticoid receptor facilitates the binding of a second transcription factor, nuclear factor 1 (NF-1) (Archer *et al.*, 1992). Cooperative binding of factors to nucleosomes has also been extensively characterised by Adams and Workman (1995).

c). Nucleosome positioning has been implicated in determining factor access. Two parameters termed translational and rotational positioning are used to describe nucleosome positions on DNA. The translational position refers to where the histone core starts and finishes on the DNA. The rotational position refers to whether the minor groove faces away or towards the histone octamer. The translational position of the nucleosome has been found to be the major determinant of the binding of TFIIA to the 5S rRNA gene (Howe and Ausió 1998). Rotational positioning has been implicated in glucocorticoid receptor binding to the MMTV promoter (Archer *et al.*, 1991; *Pina et al.*, 1990 a and b).
d). Finally, the composition of the nucleosome cores can influence factor access. For example, proteolytic removal of N-terminal tails from core histones (mimicking acetylation) was shown to enhance the binding of a GAL4 derivative to a nucleosome (Vattese-Dadey *et al.*, 1994). In addition, Lee and co-workers (Lee *et al.*, 1993) demonstrated a positive role for acetylation in TFIIA binding to nucleosomal templates *in vitro*.

Studies conducted by Jones and colleagues (Mayall *et al.*, 1997) using the T-cell receptor (TCR) α chain gene have suggested that the binding of phosphorylated CREB to chromatin templates *in vitro* can facilitate the binding of Sp1 and other T-cell specific factors to the enhancer. In addition, these investigators demonstrated that binding of phosphorylated CREB and Sp1 specifically disrupted the nucleosome array in the vicinity of the TCRα enhancer. However, to date there have been no reports concerning the interactions between CREB and a nucleosome assembled with HTLV-I LTR DNA. Therefore, in this chapter inherent binding of CREB and phosphorylated CREB to a single nucleosome was investigated.

Previous studies in this laboratory have demonstrated that the binding the bZIP protein, AP-1, to an acetylated nucleosome can completely disrupt the structure of the nucleosome *in vitro* (Ng *et al.*, 1997). As CREB is also a bZIP protein, CREB binding to an acetylated nucleosome may result in a similar disruption. Indeed, the HTLV-I model is an interesting system in which to investigate the targeting of the histone acetylase CBP to nucleosomes. The recruitment of CBP to CREB bound to an unacetylated nucleosome by Tax may directly target the nucleosome for acetylation by CBP (see figure 1.12). If CREB can disrupt an acetylated nucleosome, analogous to AP-1, then the acetylation of the nucleosome by CBP may result in disruption of the nucleosome structure, possibly aided by CREB, and be an important mechanism involved in the activation of HTLV-I gene expression.

To assemble the probes into a single nucleosome, this study employed a well characterised and widely used method of nucleosome assembly. This system involves the transfer, under high salt conditions, of a nucleosome from histone H1-depleted donor chromatin isolated from chicken red blood cells (section 2.11.1.1 and 2.11.2.1) to labelled DNA probes between 146 and 180 bp (Adams and Workman, 1995). Histones generated in this way are present primarily in the unacetylated form (Dimitrov *et al.*, 1993). In addition, the salt transfer method generates a complete single nucleosome with characteristics similar to that of native nucleosomes (Ng *et al.*, 1997). Acetylated nucleosomal templates can also be assembled by the salt transfer strategy using histones that have been purified from cells treated with sodium butyrate (section 2.11.1.4). This procedure generates nucleosomes that are largely hyperacetylated.

The probes used for the majority of the studies are the HTLV-I H147(M) and somatostatin S180 probes used in chapter 3 (table 2.3 and figure 2.1). These probes comprise the exact sequence of the naturally occurring promoters. The size of the probes (147-180 bp) ensures that only a single nucleosome can be bound to the probe however there may be differences in the translational and rotational position of the nucleosomes within the population (Luger *et al.*, 1999). As discussed in chapter 3, the use of single natural binding sites, in combination with full length recombinant proteins, represents an extensive approach to the investigation of HTLV-I transcription in a chromatin context. The characterisation of the interaction of the HTLV-I interacting proteins with a single nucleosome, by comparison to the interaction on naked DNA is an important first step in assessing the effect of chromatin on transcriptional control.

4.2 Results

4.2.1 Assembly of mononucleosomes on HTLV-I and somatostatin templates using the high salt transfer procedure

DNA templates were either assembled (or mock assembled) using the high salt transfer method as described in the methods (section 2.11.2.1). Numerous studies employing this method have been shown to routinely yield single nucleosomes on DNA fragments comprising of the standard length of 180 bp. In this work, the 147 bp probe H147(M) was used to represent the HTLV-I LTR for the majority of the studies (figure 2.1). To accurately interpret the results of gel mobility shift assays, it was desirable to have a single

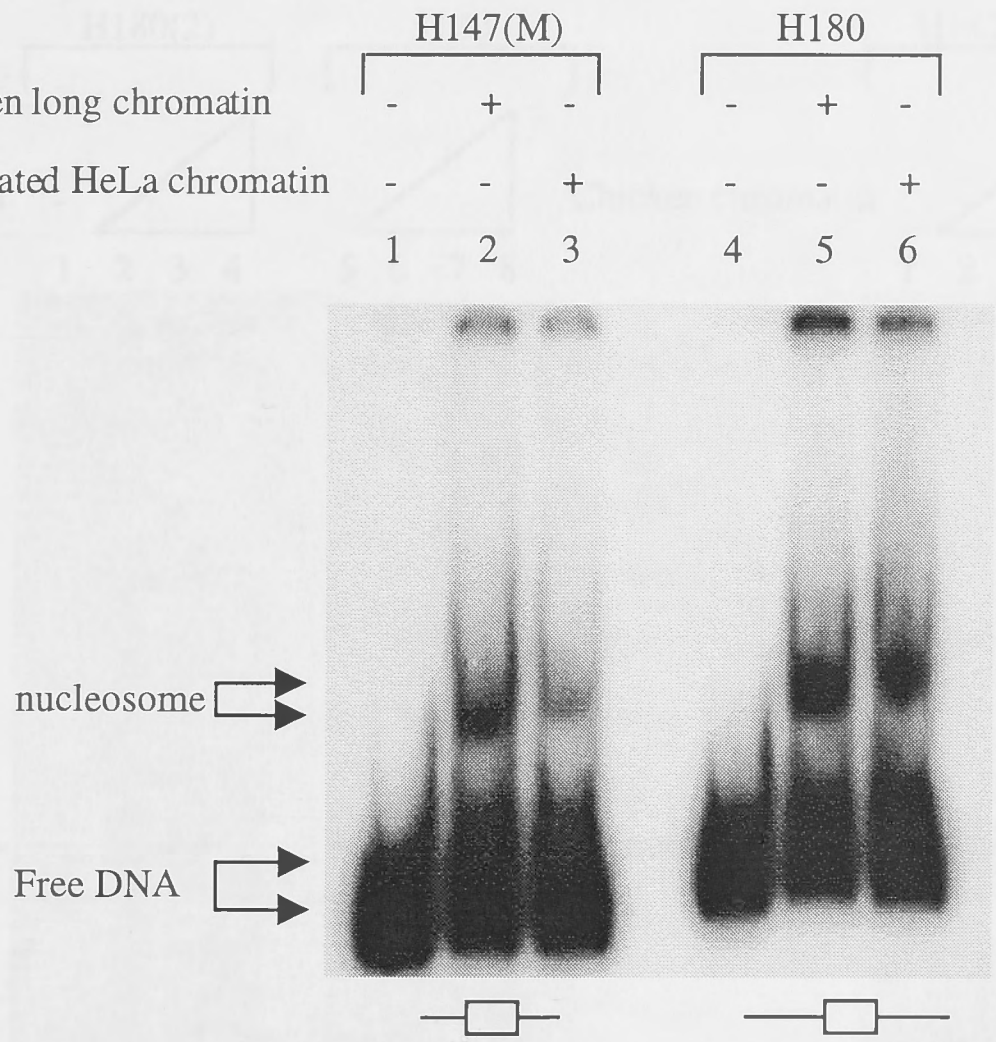


Figure 4.1: Mononucleosome assembly of the HTLV-I and somatostatin promoter probes using the salt transfer procedure. Single nucleosomes were assembled onto the HTLV-I H147(M) probe (lanes 1-3) and the somatostatin S180 probe (lanes 4-6). The formation of nucleosomes was monitored by a gel mobility shift assay. Lane 1 and 4: Free DNA. The donor chromatin used for nucleosome assembly was: Lanes 2 and 5: 50 μg chicken long chromatin; lanes 3 and 6: Acetylated HeLa chromatin. The positions of free DNA and nucleosome/DNA complexes are indicated by arrows. The position of the core TRE (Tax responsive element) or CRE (cAMP responsive element) relative to the probe is shown below the figure.

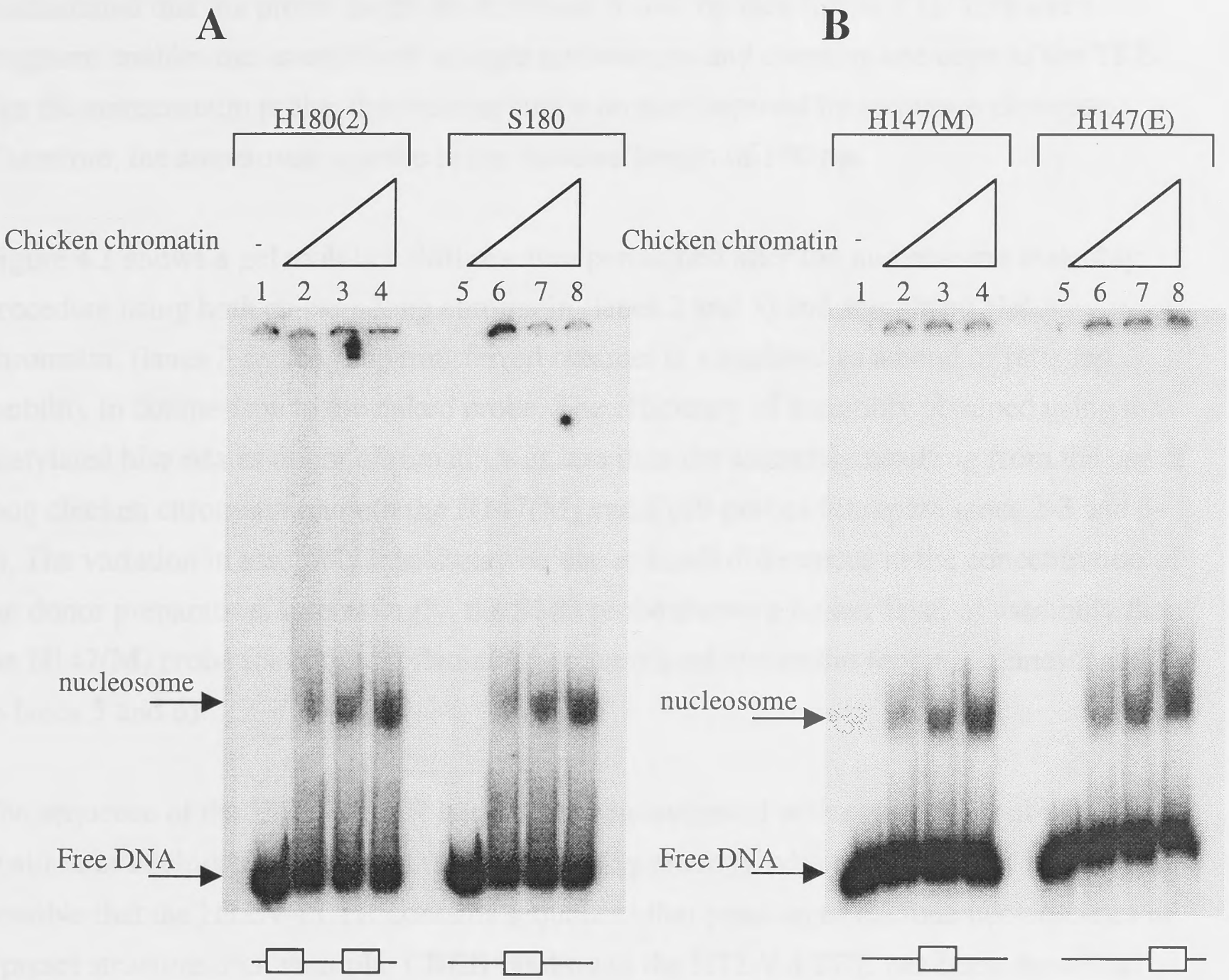


Figure 4.2: The efficiency of mononucleosome assembly depends upon the size of the probe. A. HTLV-I 180 bp probe (lanes 1-4) and the somatostatin 180 bp probe (lanes 5-8) was assembled with increasing concentrations of chicken long chromatin. The efficiency of nucleosome assembly was determined by gel mobility shift assay. lanes 1 and 5: Free DNA; lane 2-4 and 6-8: 25, 50 and 75 μg long chicken chromatin respectively. **B.** 147 bp HTLV-I probes with the core TRE element in the centre [H147(M)] (Lanes 1-4) or towards one end [H147(E)] (lanes 5-8) were assembled with increasing concentrations of long chicken chromatin. The efficiency of nucleosome assembly was determined by gel mobility shift assay. Lanes 1 and 5: Free DNA; lanes 2-4 and lanes 6-8: 25, 50 and 75 μg long chicken chromatin respectively. The positions of free DNA and nucleosome/DNA complexes are indicated by arrows. The position of the core TRE (Tax responsive element) or CRE (cAMP responsive element) relative to the probe is shown below the figure.

TRE element on the probe. The placement of the TREs on the HTLV-I sequence necessitated that the probe length be shortened to 147 bp (see figure 2.1). This sized fragment enables the assembly of a single nucleosome and contains one copy of the TRE. For the somatostatin probe, there are no limits on size imposed by sequence elements. Therefore, the somatostatin probe is the standard length of 180 bp.

Figure 4.1 shows a gel mobility shift reaction performed after the nucleosome assembly procedure using both chicken long chromatin (lanes 2 and 5) and acetylated HeLa chromatin (lanes 3 and 6). The transferred octamer is visualised as a band of retarded mobility in comparison to the naked probe. The efficiency of assembly obtained using the acetylated histones as donor chromatin was less than the assembly resulting from the use of long chicken chromatin on both the H147(M) and S180 probes (compare lanes 2-3 and 5-6). The variation in assembly levels may be due to small differences in the concentration of the donor preparation. Interestingly, the S180 probe shows a higher level of assembly than the H147(M) probe for both acetylated and unacetylated chromatin (compare lanes 2 and 3 to lanes 5 and 6).

The sequence of the HTLV-I LTR has not been investigated with regard to its ability to position or exclude nucleosomes (section 1.5). By analogy with the retrovirus HIV-1, it is possible that the HTLV-I LTR contains sequences that position or exclude nucleosomes in a preset structure. For example, CREB binding to the HTLV-I TRE has been shown to cause directed DNA bending but only in combination with the GC rich flanking sequences (Yin *et al.*, 1996). Such distortion of the HTLV-I DNA may also have a role in the positioning of nucleosomes. Since the sequence of the probe is derived form the naturally occurring HTLV-I LTR sequence, the H147(M) probe may contain sequence that excludes the formation of a nucleosome. Therefore, a possible reason for the differences in nucleosome formation between the HTLV-I and somatostatin templates shown in figure 4.1 may relate to specific sequences on the H147(M) probe. To test this possibility, a 180 bp HTLV-I probe termed H180(2) containing a double TRE element (figure 2.1) was used as the template to assemble a single nucleosome. If sequences within the H147(M) probe are responsible for decreased nucleosome assembly then the 180 bp probe should also show decreased assembly. A gel mobility shift generated from the titration of donor long chicken chromatin into the nucleosome assembly reaction containing the H180(2) and S180 probes is shown in figure 4.2 (panel A). The H180(2) and S180 probes exhibit the same level of nucleosome formation (compare lanes 2 to 4 with lanes 6 to 8). Furthermore, to test whether the central position of the TRE on the H147(M) probe effects nucleosome

assembly, a second 147 bp probe with the TRE located toward one end [H147(E)], was used as the template for nucleosome assembly. Figure 4.2 (panel B) shows that both 147 bp fragments were assembled to the same extent (compare lanes 2-3 with lanes 6-7). Therefore, the 147 bp probe assembles with less efficiency than the 180 bp probe and this differential assembly is dependent directly on the size of the template and not the presence of HTLV-I specific sequences.

4.2.2 Characterisation of CREB binding to a mononucleosome

4.2.2.1 Binding of CREB to an unacetylated mononucleosome

To examine the binding of CREB to a single nucleosome, the binding affinity of CREB for naked DNA was directly compared with its affinity for nucleosomal DNA. Figure 4.3 shows that titration of CREB onto naked DNA on both the somatostatin (panel A, lanes 2-5) and HTLV-I (panel B, lanes 2-5) probes resulted in the characteristic supershift pattern demonstrated in chapter 3. To investigate the binding of CREB to nucleosomal DNA, nucleosomes were assembled such that about 50% of the DNA template was reconstituted. (Figure 4.3, panel A, lane 6 and panel B, lane 6). The same levels of CREB as that used for naked DNA were added to the nucleosome assembled probes. Figure 4.3 panels A and B show that CREB bound preferentially to naked DNA as indicated by the disappearance of the free probe, but not the nucleosome band. Therefore a higher concentration of CREB is required to achieve nucleosome binding than that for naked DNA.

As observed for many transcription factors, addition of higher CREB concentrations to naked DNA resulted in the formation of a shifted product that migrates more slowly than the initial specific CREB-DNA complex (figure 4.4 , panel A). These oligomeric complexes probably result from protein-protein interactions with additional protein molecules. On nucleosomal templates (figure 4.4, panel B), once the free probe was completely bound by CREB, a specific complex that migrated slower than the free DNA-CREB complex (compare lanes 1 and 3) is observed when high concentrations of CREB are added to the binding reaction. This complex represents CREB bound to the nucleosome. Importantly, the demonstration of a CREB nucleosome complex shows that CREB does not completely disrupt the structure of the nucleosome but rather binds to the intact nucleosome forming a specific ternary complex containing CREB, histones and DNA. Titration of higher CREB concentrations resulted firstly in an increasing CREB nucleosome complex, followed by the majority of the protein being bound in the slowly migrating oligomeric complex (compare lanes 3, 4 and 5) similar to the oligomeric complex

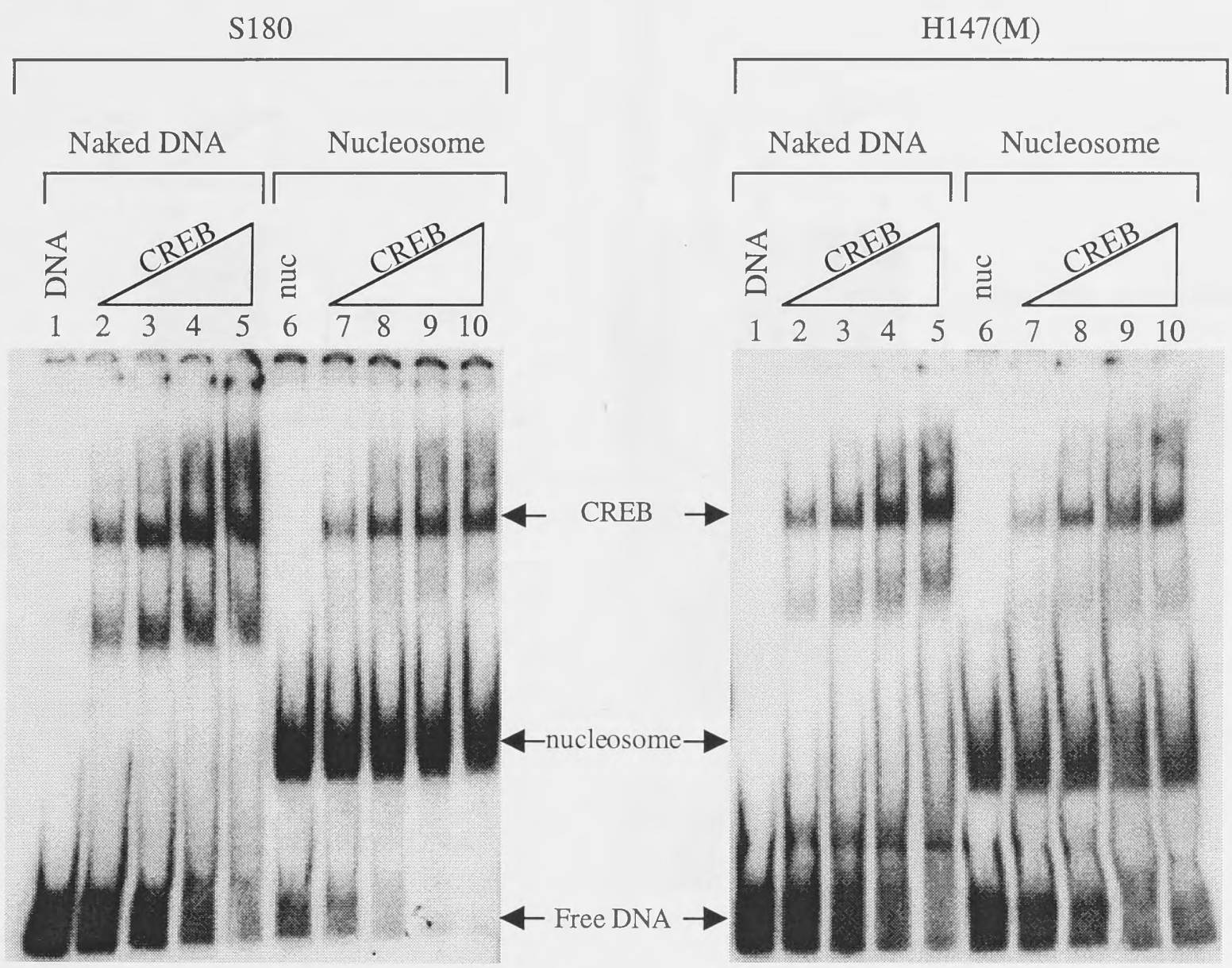


Figure 4.3: Higher concentrations of CREB are required to bind to nucleosomal DNA than are required for binding to naked DNA. The somatostatin S180 (Panel A) and HTLV-I 147(M) probes (panel B) were assembled with 50 μg of long chicken chromatin. Increasing concentrations of CREB were added in a standard binding reaction and complex formation was analysed by gel mobility shift. **A**. Binding of CREB to the somatostatin probe. Lane 1: Naked DNA; lanes 2-5 naked DNA and 1, 2, 3 and 4 μg CREB; lane 6: Nucleosomal DNA; lanes 7-10: Nucleosomal DNA and 1, 2, 3 and 4 μg CREB. **B.** Binding of CREB to the HTLV-I probe. Lanes as for panel A. The positions of free DNA, nucleosome/DNA and CREB/DNA complexes are indicated by arrows.

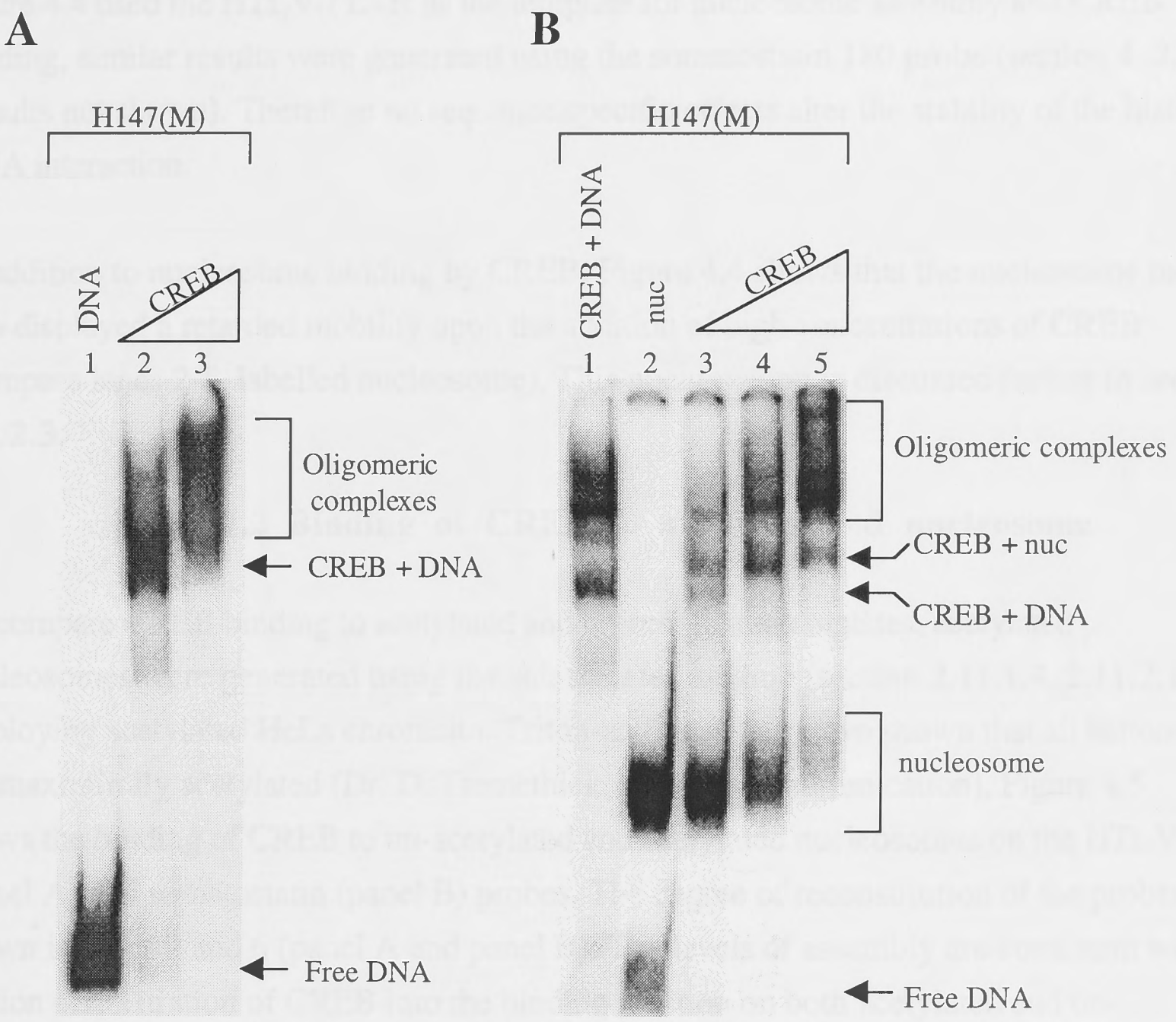


Figure 4.4: CREB binds directly to a single nucleosome assembled on the HTLV-I LTR. High concentrations of CREB were titrated into a binding reaction containing either naked DNA or DNA assembled into a single nucleosome using 50 µg chicken long chromatin. Complex formation was determined by gel mobility shift assay. **A**. Binding of CREB to naked DNA. Lane 1: Naked DNA; lane 2 and 3: Naked DNA and 3.5 µg and 7.5µg CREB respectively. **B.** Binding of CREB to nucleosomal DNA. Lane 1: Naked DNA and 3.5 µg CREB; lane 2: Nucleosomal DNA; lanes 3-5: Nucleosomal DNA and 3.5, 7.5 and 15 µl CREB respectively. The positions of Free DNA, nucleosomal DNA, CREB/DNA complexes, CREB/nucleosomal DNA complexes and Oligomeric complexes are indicated.

formed on naked DNA (compare lanes 1 and 5). CREB nucleosome binding displayed a high affinity, requiring 8 times the concentration needed to bind naked DNA. Although figure 4.4 used the HTLV-I LTR as the template for nucleosome assembly and CREB binding, similar results were generated using the somatostatin 180 probe (section 4 .2.2.2), (results not shown). Therefore no sequence specific effects alter the stability of the histone DNA interaction.

In addition to nucleosome binding by CREB, Figure 4.4 shows that the nucleosome band also displayed a retarded mobility upon the addition of high concentrations of CREB (compare lanes 2-5, labelled nucleosome). This phenomenon is discussed further in section 4.4.2.3.

4.2.2.2 Binding of CREB to an acetylated nucleosome

To compare CREB binding to acetylated and un-acetylated templates, acetylated nucleosomes were generated using the salt transfer method (section 2.11.1.4, 2.11.2.1) employing acetylated HeLa chromaitn. Triton-acid urea gels have shown that all histones are maximimlly acetylated (Dr. D. Tremethick, personal communication). Figure 4.5 shows the binding of CREB to un-acetylated and acetylated nucleosomes on the HTLV-I (panel A) and somatostatin (panel B) probes. The degree of reconstitution of the probes is shown in lanes 2 and 6 (panel A and panel B). The levels of assembly are consistent with section 4.1. Titration of CREB into the binding reaction on both acetylated and un-acetylated nucleosomes created similar mobility shift patterns (compare lanes 3-5 with lanes 7-9) . Therefore, CREB forms a stable complex with both acetylated and un-acetylated nucleosomes on the HTLV-I LTR and on the consensus CRE binding sites and histone acetylation does not facilitate CREB binding.

4.2.2.3 Binding of phosphorylated CREB to un-acetylated and acetylated nucleosomes

In chapter 3, phosphorylation was shown to increase the affinity of CREB for the HTLV-I and somatostatin probes. Therefore, the affinity of CREB phosphorylated with PKA, *in vitro* (section 2.3.1.1a), for nucleosomal HTLV-I templates was investigated. Figure 4.6 shows the binding of mock phosphorylated (panel A) and phosphorylated (panel B) CREB to un-acetylated and acetylated templates. Consistent with chapter 3 (section 3.2.1.2c), phosphorylation results in a retarded CREB-naked DNA complex (compares lanes 1 and 6, panels A and B). This same mobility shift is also evident for the CREB nucleosome

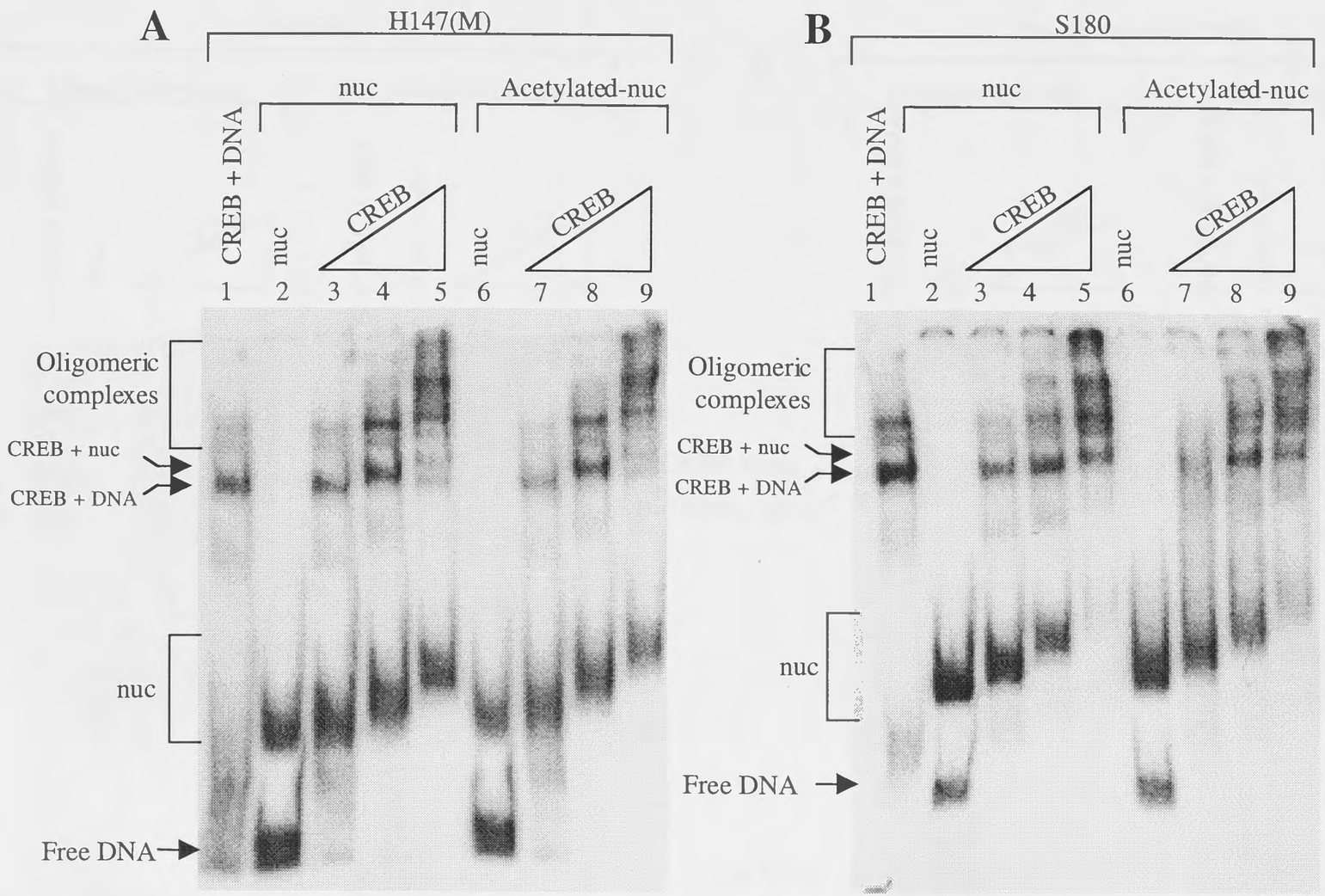


Figure 4.5: CREB binds to an acetylated nucleosome on the HTLV-I LTR and somatostatin promoters. Increasing concentrations of CREB was added to non-acetylated nucleosomes (assembled with 50 μg chicken long chromatin) or acetylated nucleosomes (assembled with 50 μg chromatin from sodium butyrate treated HeLa cells) on the HTLV-I (panel A) or somatostatin promoters (panel B). Complex formation was determined by gel mobility shift assay. **A.** Complex formation on the HTLV-I probe. Lane 1: Naked DNA and 2 μg CREB; lane 2: Nucleosomal DNA; lanes 3-5: Nucleosomal DNA and 3.5, 7.5 and 15 μg CREB respectively; lane 6: Acetylated nucleosomal DNA; lanes 7-9: Acetylated nucleosomal DNA and 3.5, 7.5 and 15 μg CREB respectively. **B**. Complex formation on the somatostatin probe. Lanes as for panel A. The positions of Free DNA, nucleosomal DNA, CREB/DNA complexes, CREB/nucleosomal DNA complexes and oligomeric complexes are indicated.

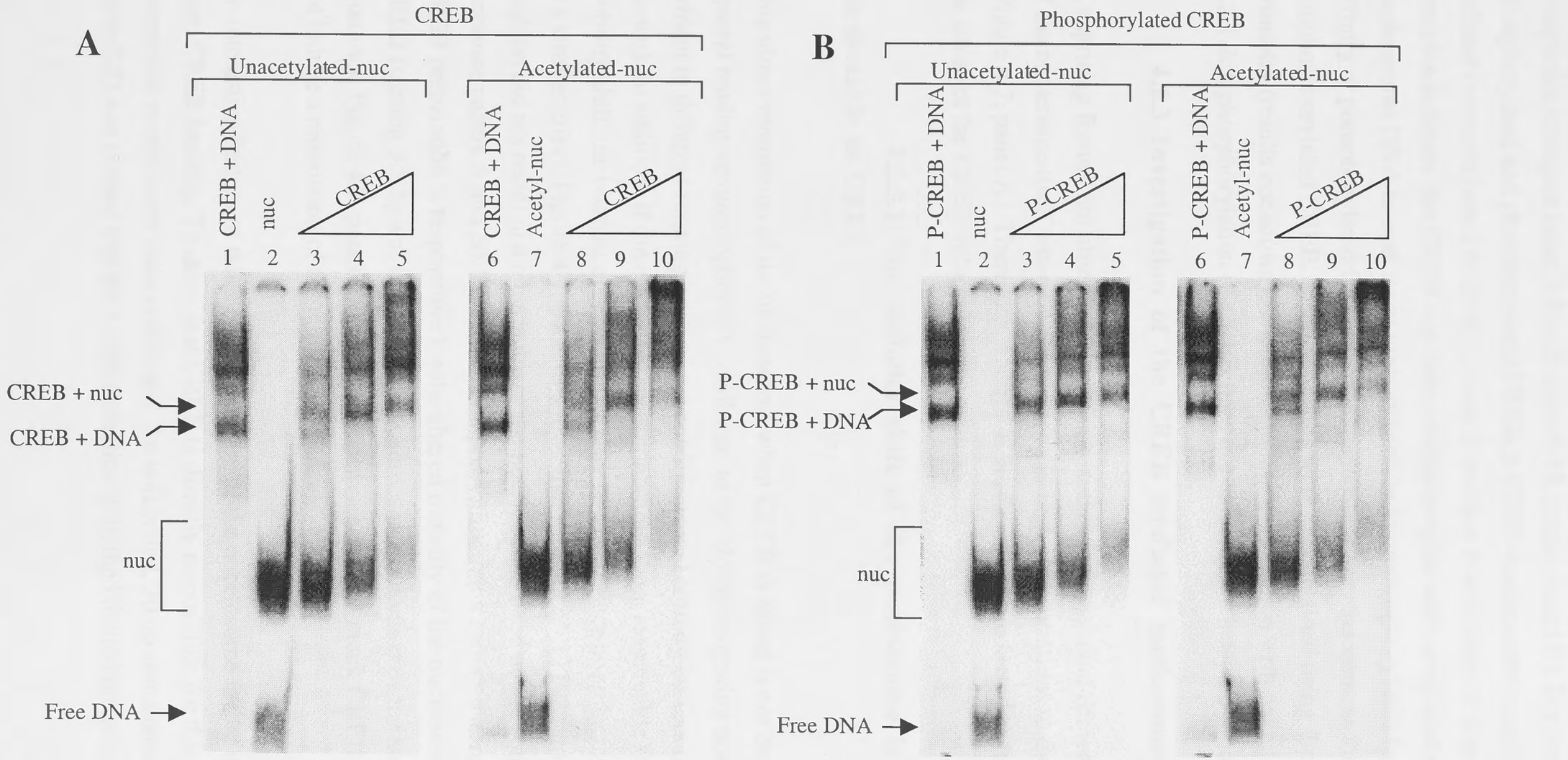


Figure 4.6: CREB and P-CREB bind to a single acetylated or non-acetylated nucleosomes with the same affinity. Increasing concentrations of CREB (panel A) or P-CREB (panel B) were added to non-acetylated (assembled with 50 μg chicken long chromatin) or acetylated (assembled with 50 μg chromatin from sodium butyrate treated HeLa cells) nucleosomes. Complex formation with the HTLV-I probe was determined by gel mobility shift. **A.** Complex formation with CREB. Lanes 1 and 6: Free DNA with 3 μg CREB; lane 2: Non-acetylated nucleosome; lanes 3-5: Non-acetylated nucleosomes with 3.5, 7.5 and 15 μg CREB respectively; lane 7: Acetylated nucleosome; lanes 8-10 Acetylated nucleosome with 3.5, 7.5 and 15 μl CREB. **B.** Complex formation with P-CREB. Lanes as for panel A except P-CREB is used in place of CREB.

complexes (compare lanes 3-5 with lanes 8-10, panels A and B). For both mock phosphorylated and phosphorylated CREB, a CREB-nucleosome complex is clearly defined (compare lane 1 to lanes 3, 4 and 5 and lane 6 with lanes 8, 9 and 10). This complex indicates that CREB can form a stable complex with acetylated or unacetylated nucleosomal DNA in both its phosphorylated and nonphosphorylated forms. However the affinity of phosphorylated CREB for the nucleosome was the same as the affinity of nonphosphorylated CREB. Identical results were also obtained using the somatostatin promoter (results not shown). Therefore, unlike the situation with naked DNA (section 3.2.1.2c), phosphorylation of CREB does not enhance nucleosome binding.

4.2.3 Investigation of the CREB mediated nucleosome mobility shift

A suprising feature not observed in previous studies is the gradual decrease in the mobility of the nucleosme itself when CREB is added in high concentrations to the binding reaction (Figure 4.7, panel A). Therefore, a series of studies were conducted to further characterise the cause of the CREB induced nucleosome retardation.

4.2.3.1 The mobility shift of the nucleosome is directly attributable to CREB

This minor retardation of the nucleosome when CREB is added is not dependent on the general binding or electrophoresis conditions as the slower migrating nucleosme was also evident in different running buffers, gel conditions and binding buffers (results not shown). In addition, if the electrophoresis conditions were responsible for the nucleosome mobility shift, the binding of ATF-2 (a closely related transcription factor), may also result in a similar effect. Figure 4.7, panel B shows that titration of ATF-2 into the binding reaction did not result in a retardation of the nucleosomal band on the somatostatin probe (compare panels A and B). To rule out the possibility that a contaminating protein in the CREB preparation is responsible for the altered mobility of the nucleosome band, mock CREB (section 3.2.1) was bound to nucleosomes in the context of the somatostatin promoter. Figure 4.7, panel C clearly demonstrates that the mock CREB preparation does not induce a retardation of the nucleosomal band.

An interesting feature of this slight retardation of the nucelosome is that it is dependent upon CREB binding. To show that CREB is directly responsible for this retardation, cold competitor experiments were conducted. An unlabelled, 30 bp somatostatin CRE probe (table 2.2) was titrated into the binding reaction with the labelled probe as described in the

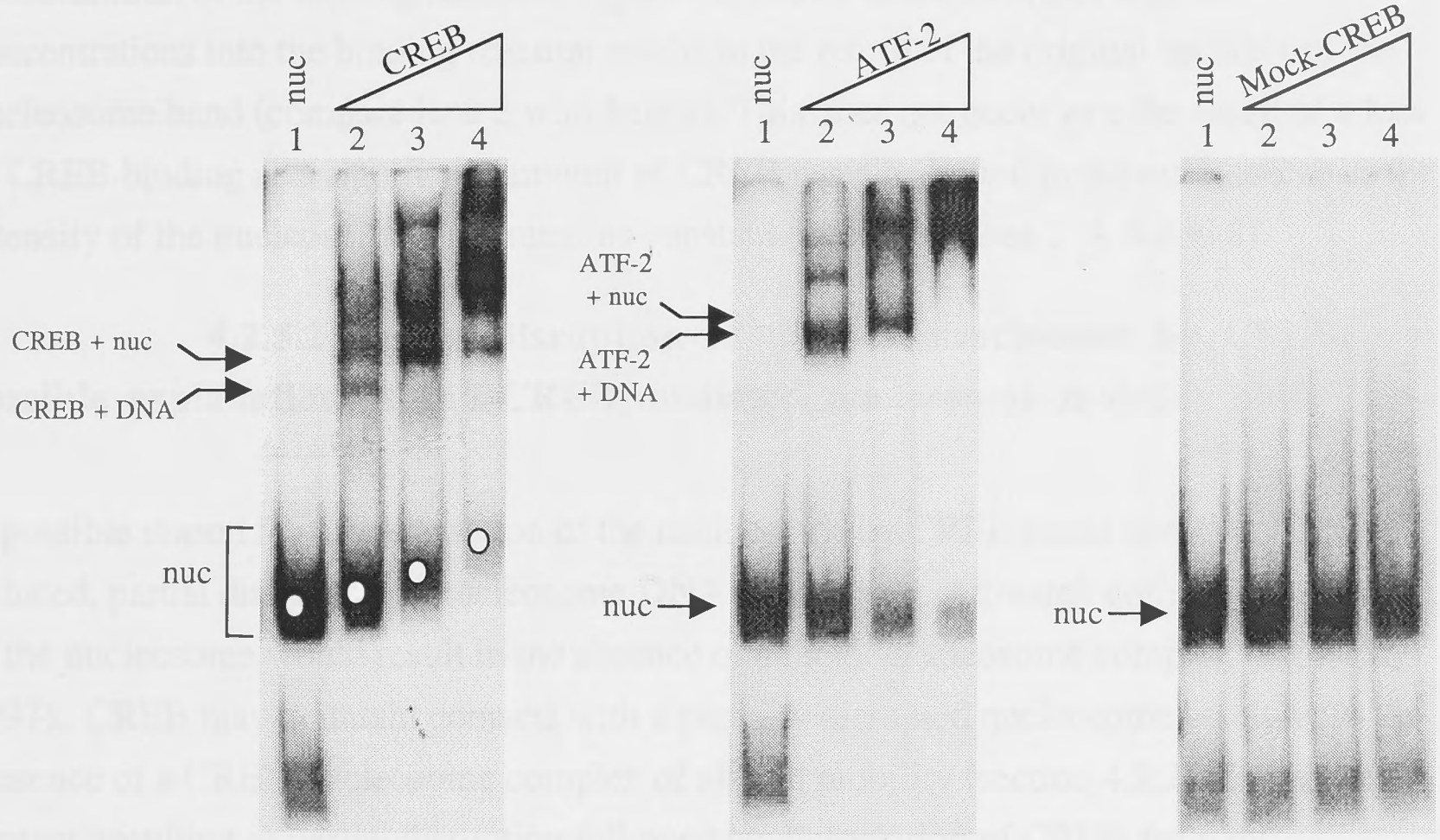


Figure 4.7: The mobility shift of the nucleosome is directly attributable to CREB. Gel shift analysis of CREB (panel A), ATF-2 (panel B) and mock CREB (panel C) bound to DNA assembled into a single nucleosome using 50µg of chicken long chromatin. **A.** Lane 1: Nucleosomal DNA; lane 2-4: Nucleosomal DNA with 3.5, 7.5 and 15 µg CREB. Retardation of the nucleosomal band is indicated by a white dot. **B.** Lane 1: Nucleosomal DNA; lanes 2-4: Nucleosomal DNA with 3.5, 7.5 and 15 µg CREB respectively. **C.** Lane 1: Nucleosomal DNA; lane 2-4: Nucleosomal DNA with 3.5, 7.5 and 15 µl mock-CREB.

methods (section 2.4.3). Figure 4.8 shows that increasing cold competitor concentration resulted in the concomitant return of the nucleosome band to its original mobility (compare lane 4 with lanes 5-10). A cold competitor control (GAL4 30 bp probe), which does not contain a CREB binding element (table 2.2), had no effect on the mobility of the nucleosome band (lanes 11-16). Therefore, this data strongly suggests that CREB is directly responsible for the retardation of the nucleosome band.

The only condition that affected the mobility of the nucleosome band was the salt concentration of the binding reaction. Figure 4.9 shows that titration of high salt concentrations into the binding reaction results in the return of the original mobility of the nucleosome band (compare lane 2 with lane 8). This does not occur as a the result of a loss of CREB binding as a significant amount of CREB remains bound to the nucleosome as the intensity of the nucleosome band remains constant (compare lanes 2, 4, 6 and 8).

4.2.3.2 Partial disruption of the mononucleosme by CREB; a possible explanation of the CREB mediated nucleosome mobility shift

A possible reason for the retardation of the nucleosome by CREB could involve a CREB induced, partial disruption of nucleosome-DNA interactions. Although complete disruption of the nucleosome would result in the absence of a factor-nucleosome complex (Ng *et al.*, 1997), CREB may maintain contacts with a partially disrupted nucleosome resulting in the presence of a CREB-nucleosome complex of altered mobility (section 4.2.2). Transient contact, resulting in partial disruption followed by dissociation of CREB from the nucleosome, could explain the retardation of the nucleosome band when the CREB concentration is not saturating. The gel-shift conditions may trap this nucleosome in an altered conformation.

a) Determination of translational position by exonuclease III digestion

To test whether the translational position of the octamer is altered by CREB, the translational positions of the retarded nucleosome was mapped by exonuclease III (ExoIII) digestion. For this procedure, CREB was bound to nucleosomal templates labelled on the 5' strand only (section 2.6.1). Since ATF-2 does not retard the mobility of the nucleosome, ATF-2 binding reactions were used as a control. After separating the nucleosome from higher order complexes by gel electrophoresis, the nucleosome band was

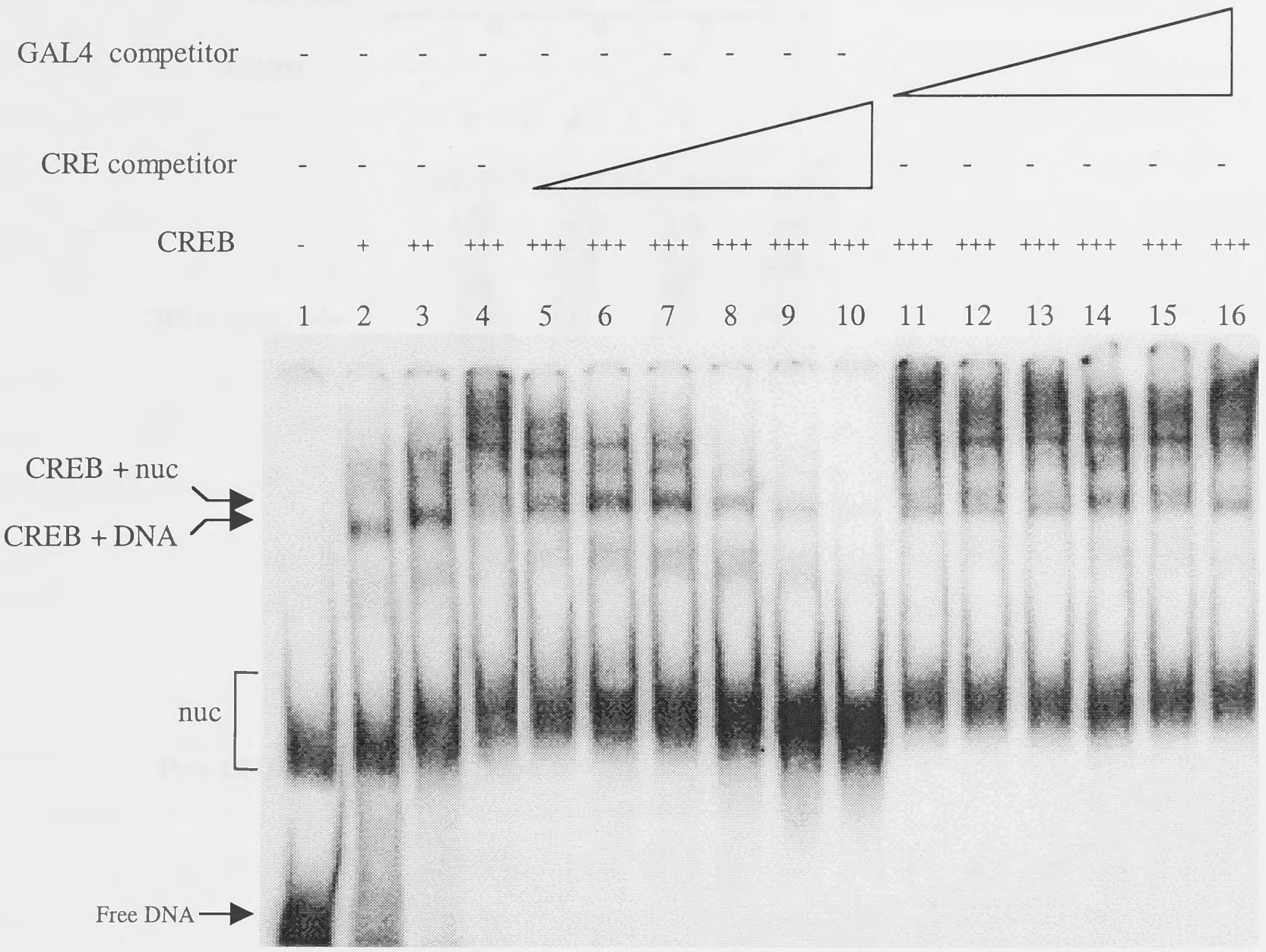


Figure 4.8: Competition of CREB away from the nucleosomal probe reduces the nucleosome mobility shift. Complex formation between CREB and the nucleosome assembled somatostatin S180 probe was competed against 100-1000 molar excess of cold CREB specific CRE probe or non-specific GAL4 probe. Lane 1: Nucleosomal DNA; lanes 2-4: Nucleosomal DNA with 3.5, 7.5 and 15 μg CREB; lanes 5-10: Nucleosomal assembled DNA with 15 μg CREB and 100, 250, 300, 500, 750 and 1000 times excess of cold CRE probe competitor; lanes 11-16: 15 μg CREB and 100, 250, 300, 500, 750 and 1000 times excess of GAL4 probe.

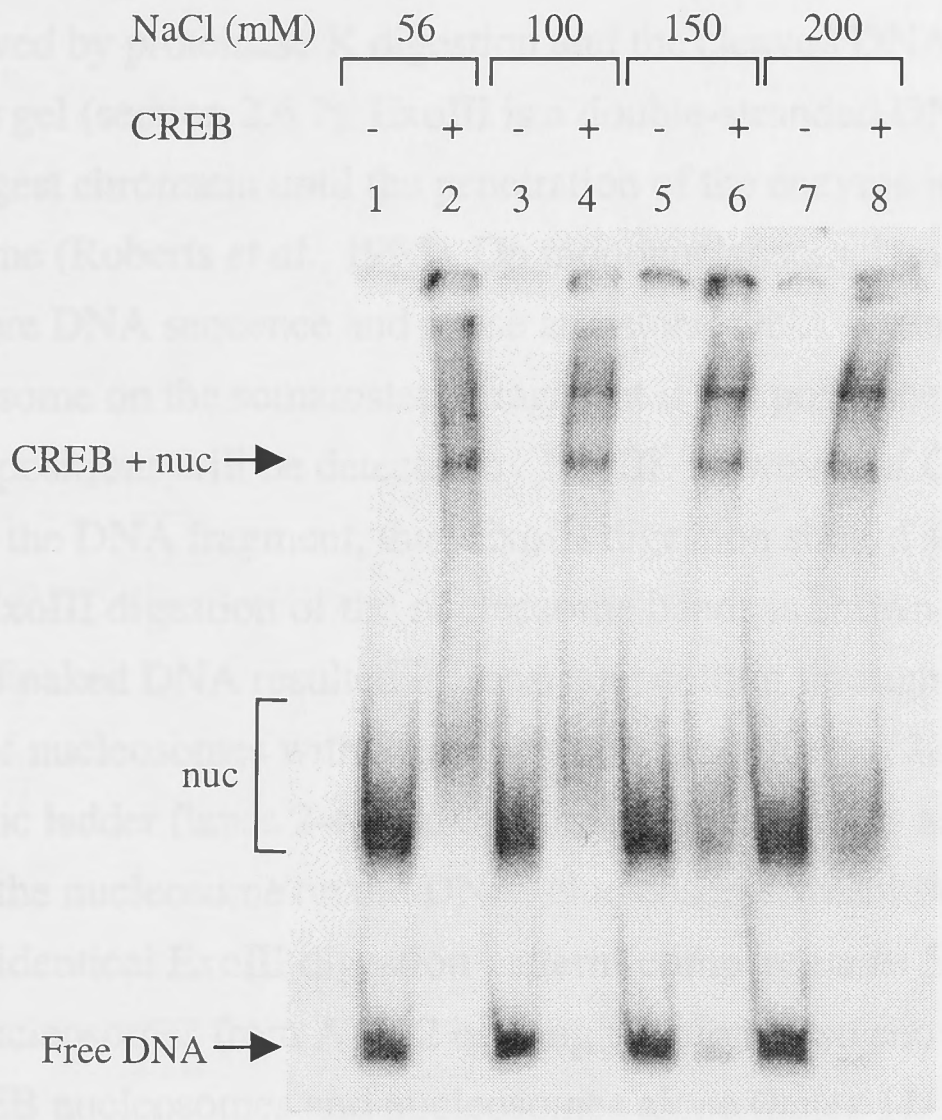


Figure 4.9: High salt concentrations inhibit the nucleosome mobility shift. CREB was bound to the somatostatin s180 probe assembled into a single nucleosome with increasing concentrations of NaCl in the binding reaction. Odd numbered lanes contain nucleosomal DNA only and even numbered lanes contain nucleosomal DNA and 7.5 μg CREB. Lane 1 and 2: 56 mM NaCl (normal salt concentration); lanes 3 and 4: 100 mM NaCl; lanes 5 and 6: 150 mM salt; lanes 7 and 8: 200 mM salt.

excised from the gel. The gel fragments were exposed to ExoIII digestion, bound proteins were removed by proteinase K digestion and the cleaved DNA was resolved on a standard sequencing gel (section 2.6.2). ExoIII is a double-stranded DNA specific 3'-exonuclease that will digest chromatin until the penetration of the enzyme is hindered by the presence of a nucleosome (Roberts *et al.*, 1995). On mononucleosomal templates, the enzyme will enter the core DNA sequence and pause at histone-DNA contact points every ten base pairs. The nucleosome on the somatostatin fragment is not positioned, therefore, a range of translation positions will be detected by ExoIII. However, if CREB does position the octamer on the DNA fragment, than ExoIII digestion should show an altered ladder. The results of ExoIII digestion of the nucleosome bands is shown in figure 4.10. ExoIII digestion of naked DNA resulted in consistent cutting throughout the sequence (lane 1). Digestion of nucleosomes with increasing concentrations of ExoIII resulted in a characteristic ladder (lanes 2-4) and the first major cut site is attributed to the boundary of the start of the nucleosome on the DNA. Nucleosomes resulting from CREB interaction showed an identical ExoIII digestion pattern (compare lanes 5-7 and 8-9 with lanes 2-4). In addition, nucleosomes from ATF-2 binding reactions also showed an identical pattern to that of CREB nucleosomes and nucleosomes alone (lanes 11-16). Therefore, CREB does not appear to alter the translational position of the octamer as qualitatively, there is no change in nucleosome position, and quantitatively, there is no indication of an altered nucleosomal structure.

b) Cross-linking of histones to DNA

In order to confirm the results obtained using ExoIII, the technique of DNA cross linking was also employed to investigate the CREB-nucleosome mobility shift. If CREB binding to the nucleosome does result in partial disruption or alteration of the conformation of the histone octamer, then prior cross-linking of the octamer to the DNA would prevent such a disruption and no retardation of the nucleosome band would be observed. Therefore, two cross-linking strategies were employed. Firstly, nucleosomes were cross-linked to DNA using formaldehyde as described in the materials and methods (section 2.7.1). Secondly the cross-linking agent dimethyl suberimidate was utilised to cross-link proteins within the histone octamer (section 2.7.2). Figure 4.11 (panel A), shows a silver stained SDS-PAGE of cross-linked and mock cross-linked histones. Cross-linking by dimethyl suberimidate results in the formation of a complex with a slower mobility than the free histones (compare lanes 1 and 2). In addition, the histones H2A and H2B are completely removed from the free histones. Cross-linking of the histones to DNA by formaldehyde results in a complex

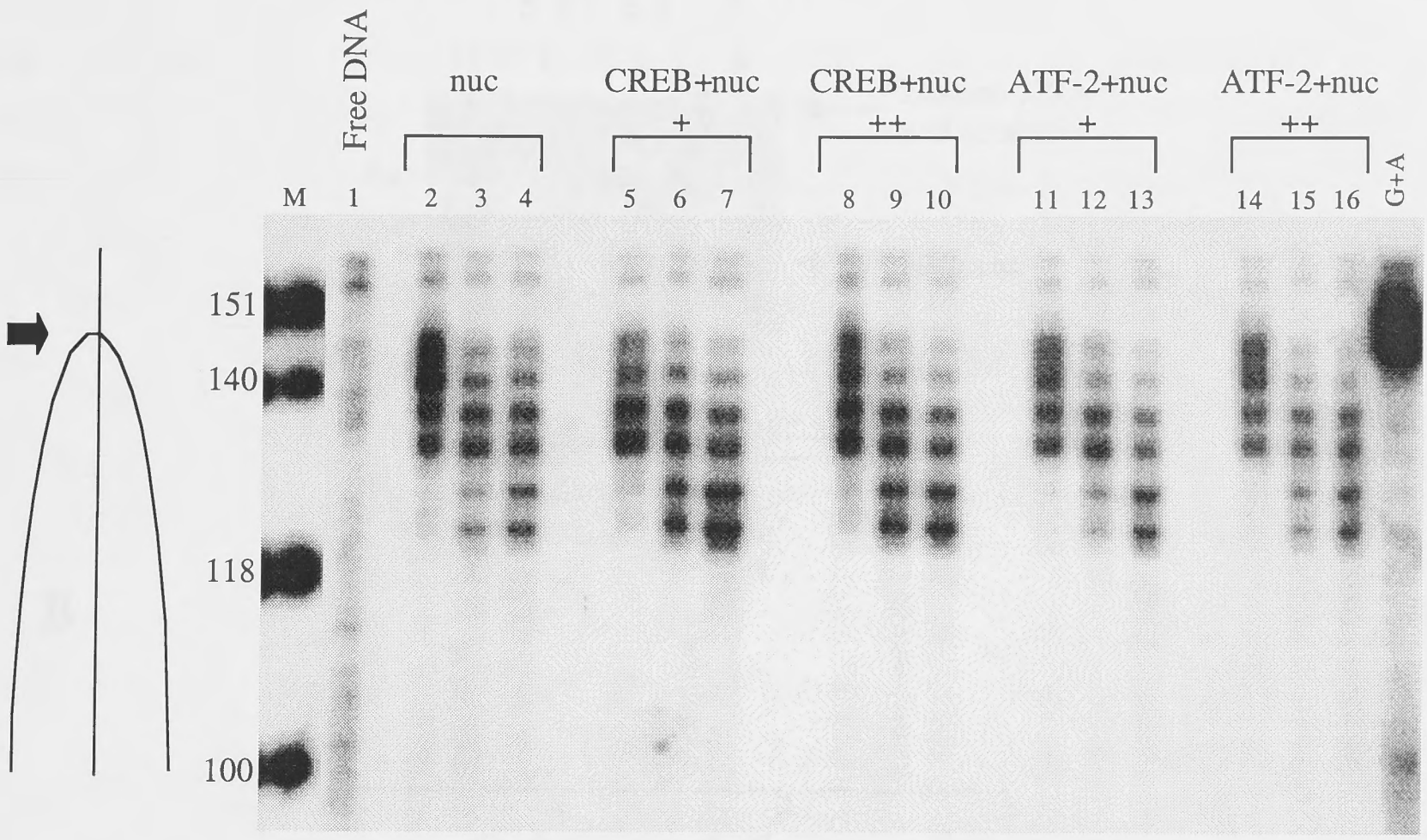


Figure 4.10: Exonuclease III digestion of a single nucleosome. 3.5 μg or 7.5 μg of CREB or ATF-2 was bound to a single nucleosome assembled on the somatostatin s180 probe. The complexes were resolved by gel mobility shift. The nucleosomal complex or free DNA was excised form the gel and digested with increasing concentrations of Exonuclease III (Exo III). The digestion products were resolved on a 8%, 7M urea sequencing gel. Lane 1: Free DNA (control); lanes 2-4: Nucleosomal DNA control; lane 5-7: Nucleosomal DNA with 3.5 μg CREB; lane 8-10: Nucleosomal DNA with 7.5 μg CREB; lane 11-13: Nucleosomal DNA with 3.5 μg ATF-2; lanes 14-16: Nucleosomal DNA with 7.5 μg ATF-2. DNA molecular weight markers (bp) are shown on the left and the G+A ladder for the somatostatin probe is shown on the right. Although the nucleosomes are not positioned, the arrow indicates the position of the first nucleosome DNA boundary.

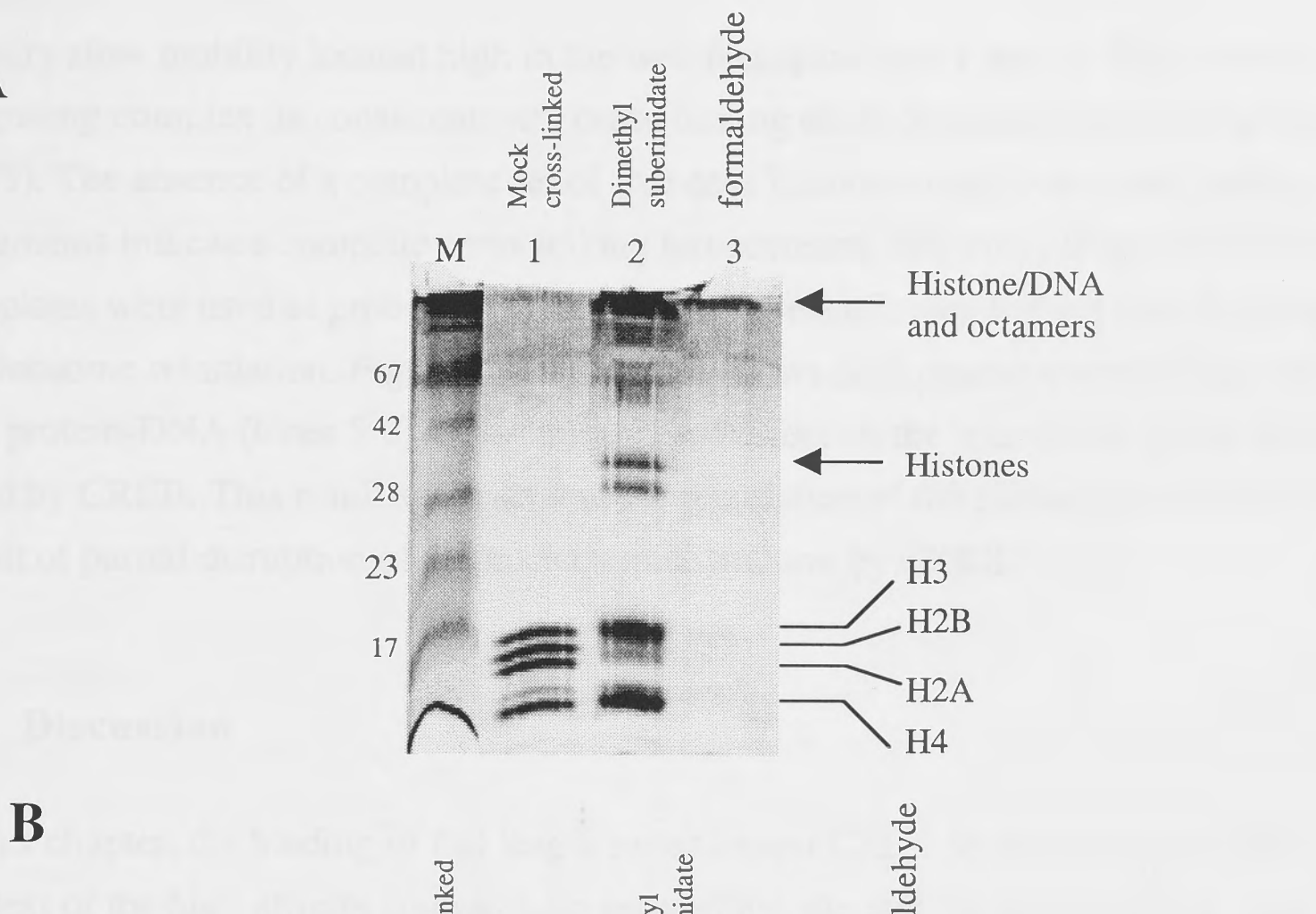


Figure 4.11: Cross-linking has no effect on the mobility shift of the nucleosome band. A. Analysis of cross-linked nucleosome cores. Silver stained 15% SDS-PAGE of mock cross-linked histones (lane 1), nucleosomes cross-linked by dimethyl suberimidate (protein-protein), (lane 2) and nucleosomes cross-linked by formaldehyde (protein-DNA), (lane 3). The position of the four core histones, the cross-linked histone dimers, cross-linked histone octamers and DNA octamer cross-linking are indicated. Molecular weight markers (kDa) are shown at the left of the figure. **B.** Increasing concentrations of CREB were bound to mock cross-linked nucleosomal DNA (lanes 1-4), dimethyl suberimidate cross-linked nucleosomal DNA (lanes 5-8) and formaldehyde cross-linked nucleosomal DNA (lanes 9-12) shown in panel A. Complex formation was resolved by gel mobility shift analysis. Lanes 1, 5 and 9: Nucleosomal DNA; lanes 2-4, 6-8 and 10-12: Nucleosomal DNA with 3.5 μg CREB, 7.5 μg CREB and 15 μg CREB respectively.

of very slow mobility located high in the well (compare lane 1 and 3). This slowly migrating complex is consistent with cross-linking of the histones to DNA (Jackson, 1999). The absence of a complete set of free core histones using both cross-linking treatments indicates complete cross linking has occurred. The cross-linked nucleosomal templates were used as probes to investigate the effect of cross-linking on CREB induced nucleosome retardation. Figure 4.11 (panel B) shows both protein-protein (lanes 10-12) and protein-DNA (lanes 5-8) cross-links had no effect on the retardation of the nucleosome band by CREB. This result suggests that the retardation of the nucleosome band is not the result of partial disruption of the nucleosome structure by CREB.

4.3 Discussion

In this chapter, the binding of full length recombinant CREB to nucleosomal DNA in the context of the high affinity somatostatin recognition site and the lower affinity viral TRE was investigated. The results from the binding studies carried out in this chapter demonstrate that under the *in vitro* binding conditions described in section 2.4, recombinant CREB can bind to its recognition sites (CRE or TRE) on the surface of the nucleosome positioned over the binding site with a relatively modest reduction in binding affinity (about 8 fold). Published studies have shown that other transcription factors such as TFIIA , glucocorticoid receptor, GAL4 derivatives, USF, Myc/Max, Sp1, NFκB and AP-1 can bind to nucleosomal DNA *in vitro* (Adams and Workman, 1995; Chen *et al.*, 1994; Lee *et al.*, 1993; Li and Wrange, 1995; Perlmann and Wrange, 1998, Rhodes, 1985; Taylor *et al.*, 1991, Wechsler *et al.*, 1994; Ng *et al.*, 1997).

This study confirms and extends work by Myall *et al.* (1997) who showed a similar ability of CREB to bind nucleosomal DNA. Although, in the studies conducted by these investigators, CREB was incubated with the DNA template (the TCRα enhancer) during nucleosome assembly. Therefore their results may reflect competition between CREB and the octamer for DNA and not binding of CREB to the nucleosome. The work in this chapter extends these results by showing that CREB binds to its recognition site in a pre-assembled nucleosome. Furthermore, we have demonstrated that CREB binds its recognition site on nucleosomal templates with only a 7-8 fold less affinity than to a corresponding site on naked DNA. This puts CREB in the category of strong nucleosome-binding transcriptional activators which includes the glucocorticoid receptor (GR) and AP-

1. GR binds to the nucleosomal glucocorticoid response element (GRE) with a 2-8-fold reduction in binding relative to naked DNA with the GRE held in certain translational and rotational positions (Li and Wrange, 1995). AP-1 also has a high affinity for nucleosome DNA (Ng *et al.*, 1997) and therefore nucleosome binding may be a characteristic of leucine zipper proteins. The nucleosomal templates used in this chapter exhibit heterogeneity with regard to the rotational and translational position as ExoIII digestion did not result in a 10 bp ladder (section 4.2.3.1). Therefore it is possible that different binding affinities of CREB for the nucleosome also exist that reflect differences in the rotational position of the CRE on the surface of the nucleosome. However the relative contribution of binding site position on the nucleosome is beyond the scope of this thesis.

A number of published studies have investigated the interactions between transcription factors and nucleosomes. However, many of these studies have employed artificial components. For example, promoter sequences, often in the form of tandem repetitions of multiple binding sites are used as the template for nucleosome assembly and factor binding. Furthermore artificial factors in the form of truncated proteins such as the GAL4 derivative GAL4-AH or chimeric proteins such as GAL4-VP16 are often used in combination with the artificial promoter sequences (Taylor *et al.,* 1991; Vettese-Dadey *et al.*, 1994; Li and Wrange 1993, 1995). Artificial promoters are useful as introduced sequences can hold nucleosomes in fixed translational and rotational positions to precisely define nucleosome factor interactions as is the case for the glucocorticoid receptor (Li and Wrange 1995). In addition, the use of multiple binding sites and artificial factors can increase the affinity of the factor for the nucleosome. For example GAL4-AH can bind to its recognition site with a 100-fold decrease in affinity for a single site (relative to naked DNA) and only a 10-fold decrease in affinity for five consecutive sites (Taylor *et al.*, 1991). Importantly, this study demonstrates that full length CREB can bind to both a high affinity site (somatostatin CRE) and a low affinity site (HTLV-I TRE) on natural promoter sequences. Therefore, the relatively high affinity of CREB for the nucleosome *in vitro* may more closely resemble the *in vivo* situation.

Although nucleosome formation results in a change in accessibility of factors for their recognition sites on DNA, histone-DNA interactions may affect other properties of DNA-factor interactions within the nucleosome. For example, chapter 3 showed that phosphorylation of CREB resulted in an increase in binding affinity for its recognition site on naked DNA. However, in this chapter, the phosphorylation state of CREB has no effect on its ability to bind a nucleosome. Although it is possible that the effect of

phosphorylation is masked by the higher concentrations of CREB required for nucleosome binding.

In addition to altering the accessibility of factors for DNA, nucleosome formation also dramatically alters the conformation of DNA (section 1.2.1). The bending of naked DNA, as the result of intrinsic bending sequences or factor binding, has been the subject of many investigations. For example, the somatostatin promoter has been shown to have an intrinsic bend that is straightened by CREB binding (Paotella *et al.*, 1994; Yin *et al.*, 1995). In addition, binding of different bZIP protein family members has been shown to differentially bend DNA (Kerppola and Curran, 1991; 1993; Kerppola, 1996). Since reconstitution of DNA into nucleosomes alters the conformation of the DNA via tight DNA bending, the effect of sequences and factors on DNA bending must be re-evaluated within a nucleosome context. For example, in this study, the affinity of CREB for both the high affinity CRE and low affinity TRE DNA binding sites was reduced by an equal amount (8 fold) when these sites were incorporated into a nucleosome. On naked DNA, the difference in affinity of CREB binding to the CRE and TRE sites has been attributed to the single base pair change in the core element (see figure 1.8B) and the presence of GC rich flanking sequences on the HTLV-I LTR (section 1.8.3). It has been suggested by some groups that the flanking GC sequence present on the HTLV-I TRE, but not on the somatostatin CRE, creates a bend in the naked DNA which affects CREB binding (Yin *et al.*, 1996). In this study we have shown that bending of the DNA around the nucleosome reduces CREB binding by the same extent regardless of the presence of GC sequences. On nucleosomal DNA, unless the GC-induced bend is conserved on the nucleosome, the difference in core sequence is more likely to be the cause of the affinity difference between the CRE and TRE elements rather than bending of the flanking GC rich sequences.

In this study, we showed that high concentrations of CREB in the binding reactions in addition to a nucleosome-CREB supershift, caused a minor retardation of the nucleosome band. This effect can be can be directly attributable to CREB through a number of lines of evidence. The mock CREB protein preparation had no effect on the mobility of the nucleosome. In addition, titration of a 30 bp CRE cold competitor into the binding reaction resulted in the dose dependent removal of CREB from the 180 bp nucleosome template and a concomitant return of the original mobility of the nucleosome. Furthermore, the binding or electrophoresis conditions (except for high salt) used in this study were not responsible for the mobility change as alternative conditions had no effect on the mobility shift by

CREB. In addition, the binding of a similar protein ATF-2, also a member of the CREB/ATF transcription factor family, had no effect on nucleosome mobility using the same binding conditions used for CREB.

One possible explanation for this observation is that CREB binding to the nucleosome partially disrupts the structure of the nucleosome or alters its translational position. The retarded nucleosome band may arise from the partial disruption of the nucleosome followed by dissociation of CREB from the nucleosome complex during electrophoresis. The change in mobility of the higher-order CREB-nucleosome complexes may be the result of CREB remaining bound to a partially disrupted nucleosome. Partial disruption of the nucleosome could cause in a change in mobility of the complex analogous to the phosphorylation mediated change in CREB mobility shown in chapter 3 section (3.2.1.2c). A change in the translation position of the nucleosome mediated by CREB could also change the mobility of the nucleosome (Luger *et al*., 1999) after CREB dissociation.

ExoIII digestion has been used by many investigators to map the translational positions of mononucleosomes (Blomquist *et al*., 1996; Li and Wrange, 1993; 1995; Roberts *et al*., 1995). In this study we used ExoIII digestion to compare the translational positions of the retarded nucleosome to a nucleosome of normal mobility. The pattern generated by ExoIII digestion of the nucleosomes of differing mobilities showed identical patterns. Therefore it is unlikely that CREB significantly alters the translational position of the mononucleosome. This result was further confirmed by the technique of cross-linking. Cross-linking of nucleosomes to DNA has been extensively used to study nucleosome dynamics (Jackson, 1999). In addition, protein-protein cross-linking within the histone octamer has been successfully used to show that octamer disruption is required for the stimulation of transcription factor binding (Walter, *et al* ., 1995). In this chapter, cross-linking was employed as a tool to determine whether CREB can partially disrupt the nucleosome. However, we have been unable to detect evidence supporting this hypothesis as cross-linking of the histone octamer to the DNA or protein-protein cross-links within the octamer had no effect on the CREB induced change in nucleosome mobility.

The reason for this CREB induced change in mobility of the nucleosome currently favoured by this laboratory is that a break-down product of CREB, resulting from the purification process, binds to the nucleosome and retards its mobility. The presence of a minor band that migrates with slower mobility in gel mobility shifts is consistent with the presence of a break-down or truncated CREB product. The presence of such a product has also been

noted by other investigators (Giebler *et al.*, 1997). The break-down product may exhibit lower binding affinity than full length CREB as the nucleosome shift is only evident at high CREB concentrations where full length CREB is bound to the nucleosome. This hypothesis is additionally supported by the observation that a salt concentration four times that of normal binding conditions inhibits the nucleosome retardation. Addition of salt to the binding reaction results in the return of naked DNA, whereas the CREB-nucleosome band and the nucleosome band remain at the same intensity (see figure 4.9). This indicates that the high ionic strength may inhibit interactions between the break down product and nucleosomes while enabling full length CREB to remain bound to the nucleosome. In order to test this possibility it would be interesting to label the CREB preparation with a fluorescent dye to monitor the positions of CREB in the gel shift. If a CREB break-down product does bind to the nucleosome than it should be detected by this method.

In addition to identifying CREB as a nucleosome binding factor, an important finding in this chapter is that the binding of CREB alone to nucleosomal templates whether acetylated, or un-acetylated, is not sufficient to bring about chromatin remodelling effects such as nucleosome disruption or displacement on the HTLV-I LTR. CREB instead forms a specific complex with the nucleosome, as supported by the detection of a nucleosome-CREB complex in gel mobility shift assays (section 4.2.2). Therefore other components and/or mechanisms, besides CREB binding are required to facilitate the disruption or displacement of the nucleosome. Previous studies in this laboratory have demonstrated that a related leucine zipper transcription factor, AP-1 can completely disrupt the structure of an acetylated nucleosome. The inability of CREB to disrupt the nucleosome is surprising as AP-1 and CREB are both members of the bZIP protein family and share similar DNA binding motifs (Ellenberger *et al.*, 1992) (figure 4.12).

The structure of the yeast bZIP factor GCN4, bound to DNA, has been determined by x-ray crystallography (Keller *et al.*, 1995; König and Richmond, 1993). Analysis of this structure shows that DNA contacts are mediated by a distinct set of amino acid residues. However, alignment of the GCN4 structure with that of other bZIP proteins indicates that these residues are conserved throughout the bZIP family regardless of the binding site (figure 4.12). The involvement of these residues in DNA interactions has been confirmed by the solving of the structure of the Fos-Jun heterodimer-DNA complex (Chen *et al.*, 1998). Therefore the structural basis of DNA-binding specificity among the bZIP family remains unclear. It has been suggested by some investigators that bending of the DNA

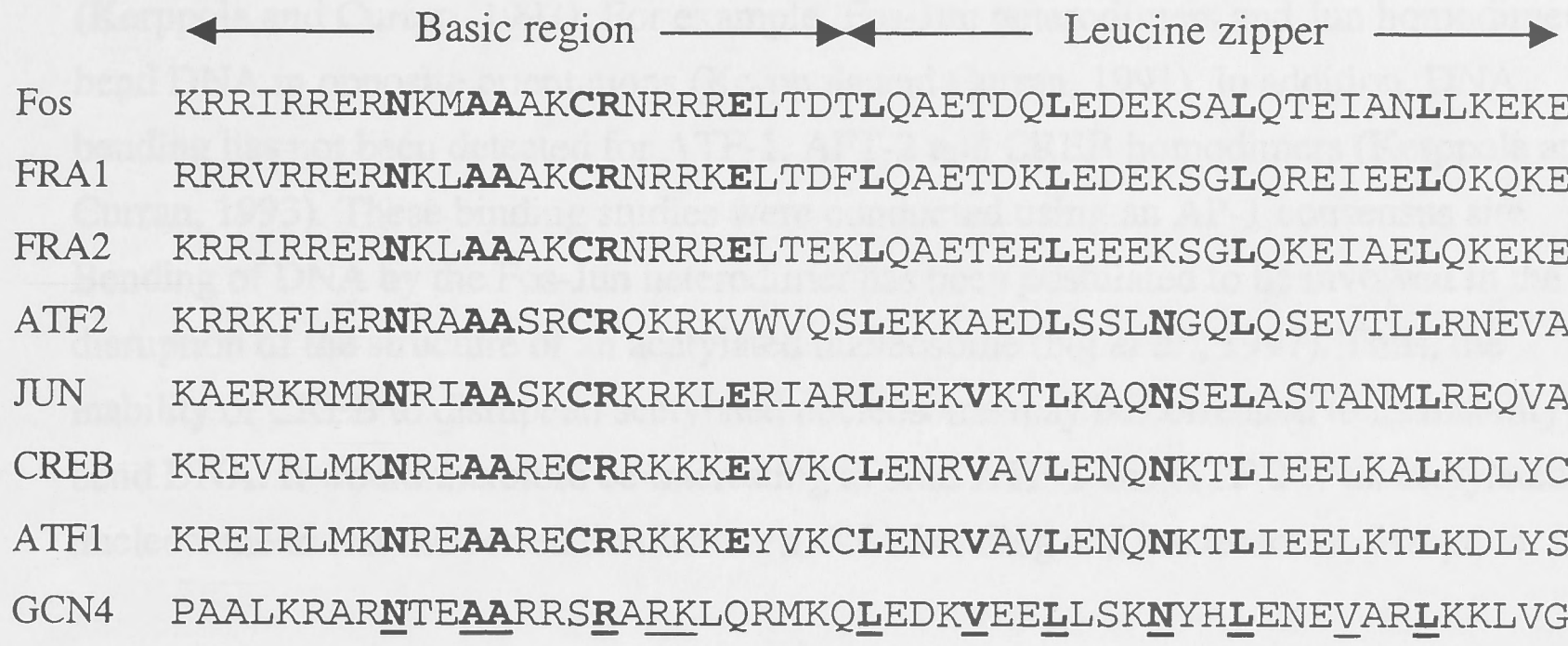


Figure 4.12: Sequence alignment of the basic region and leucine zipper of selected bZIP proteins. Residues shared among most bZIP family proteins are shown in bold. Residues identified by Konig and Richmond, (1993) as being involved in base specific GCN4 protein-DNA contacts are underlined.

induced by factor binding could be a means of discrimination between binding sites (Kerppola and Curran, 1993). For example, Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations (Kerppola and Curran, 1991). In addition, DNA bending has not been detected for ATF-1, AFT-2 and CREB homodimers (Kerppola and Curran, 1993). These binding studies were conducted using an AP-1 consensus site. Bending of DNA by the Fos-Jun heterodimer has been postulated to be involved in the disruption of the structure of an acetylated nucleosome (Ng *et al.*, 1997). Thus, the inability of CREB to disrupt an acetylated nucleosome may be correlated to its inability to bend DNA. It would therefore be interesting to bind ATF-1 and ATF-2 to an acetylated nucleosome to test the correlation between DNA bending and nucleosome disruption.

The sequence of the binding element may also affect the ability of the various factors to bend DNA. For example, binding of GCN4 to the pseudosymeteric AP-1 site (ATGA$^{C}/_{G}$TCAT) bends DNA by 10° relative to its binding to the symmetric CREB/ATF (ATGAC/GTCAT) site (Keller *et al.*, 1995). It would therefore be interesting to determine whether the Fos-Jun heterodimer can disrupt an acetylated nucleosome encompassing the CREB/ATF site on the somatostatin or HTLV-I promoters. In addition, if CREB can bend AP-1 DNA in a similar way to GCN4, than it would also be interesting to determine whether CREB can disrupt an acetylated nucleosome encompassing the AP-1 site.

Yin *et al.* (1996) demonstrated that CREB binding the HTLV-I TRE in the context of the GC rich flanking sequences results in directed DNA bending. Whereas, CREB binding to a consensus CRE element without the GC flanking sequences does bend the DNA. If DNA bending by nucleosome-factor interaction results in nucleosome disruption, than CREB may be expected to disrupt the HTLV-I LTR. However, in this study we were unable to show any disruption of the HTLV-I nucleosome by CREB as indicated by a defined CREB-nucleosome super shifted complex. An alternative explanation for the difference between CREB and AP-1 with regard to nucleosome disruption may involve direct protein-protein interactions between the factors and the histone octamer. For example, Ng *et al.* (1997) has suggested that Fos-Jun may interact directly with the H2A-H2B dimer. Thus, CREB may not interact with the octamer, or interacts with the octamer in a different way. In addition, the possibility that differences in experimental methodology (such as the method of assembly) affects the stability of the nucleosome cannot be discounted.

Several models can be put forward to accommodate the ability of CREB to activate transcription from chromatin templates (Mayall *et al.*, 1997) while not being directly

responsible for the disruption or displacement of the nucleosome. CREB may interact with chromatin modifying complexes such as ATP dependant chromatin remodelling machines (equivalent to SWI/SNF, NURF or CHRAC) or acetylating complexes (equivalent to SAGA). Targeting of these complexes to nucleosomes by CREB may cause the disruption of chromatin structure resulting in formation of functional transcriptional machinery. Evidence to support this notion comes from Xing *et al.* (1995). This group has demonstrated that CREB can interact with transcription factors TFIIB and TFIID. Importantly the interaction with TFIID was not through TBP but through co-factors in the TFIID holoenzyme. Many of the cofactors associated with TFIID are also components of the SAGA complex (see section 1.4.2.3b). Furthermore, CREB was shown to activate transcription from the TCRα gene in the presence of the *Drosophila* S-190 extract which contains many factors involved in the transcription process including chromatin remodelling factors (see chapter 5).

An alternative mechanism by which nucleosome binding by CREB results in nucleosome disruption or displacement may be through the recruitment of CBP. In the context of the HTLV-I LTR, Tax has been shown to recruit CBP to unphosphorylated CREB *in vitro* (Kwok *et al.*, 1996). Perhaps the recruitment of CBP to the HTLV-I promoter by Tax, and to cellular genes by phosphorylated CREB, results in transcriptional activation by the functions of CBP. CBP is known to interact directly with basal transcriptional machinery and to be a histone acetyltransferase. Testing of this hypothesis is the central aim of this thesis and is the subject of intensive investigation in the following chapters.

Although CREB appears established in the literature as an important cellular transcription factor required for the transcription of HTLV-I, the interaction of CREB with chromatin on the HTLV-I LTR as not been investigated. This study is the first to identify CREB as a high affinity nucleosome binding transcription factor. As transcription factor binding to chromosomal templates is the first step in the inducible transcription process *in vivo*, the ability of CREB to bind a nucleosome *in vitro* may represent the initial step in transcription of HTLV-I. Although we have identified CREB as a nucleosome binding transcription factor, the functional significance of this finding must be defined in terms of transcription.

CHAPTER 5:

The development of *in vitro* transcription and chromatin assembly systems

5.1 Introduction

The regulation of gene expression is most commonly achieved by controlling transcription. Transcription can be regulated at many steps including preinitiation, initiation, elongation and termination (Roeder, 1996). *In vivo*, eukaryotic genomes are assembled into nucleosomes and this assembly inhibits transcription by preventing the binding of RNA polymerases, other basal transcription factors and promoter specific transcription factors (Grunstein 1990; Felsenfeld, 1992). In addition, nucleosomes are also thought to block the elongation of RNA polymerase II holoenzyme (Sathyanarayana *et al.*, 1999; Chang and Luse, 1997). However, the role of chromatin structure in transcriptional regulation is likely to be more complex than a matter of repression. For example, the packaging of DNA into architecturally distinct chromatin structures appears to facilitate transcription by promoting interactions among transcription factors (Barton and Emerson 1994; Schild *et al.*, 1993; Kamakaka *et al.*, 1993).

Histones can facilitate or inhibit the transcription process via protein-protein interactions or by playing an architectural role (Grunstein *et al.*, 1995; Wolffe, 1994). Moreover, the function of histones themselves are regulated by protein modifications such as acetylation and phosphorylation, which indicates that histones are targets for cell signalling pathways (Strahl and Allis, 2000 Wade and Wolffe, 1997). To determine the precise molecular details of how chromatin regulates transcription, and how the structure of chromatin itself is regulated, it is essential to mimic the *in vivo* transcriptional activation process *in vitro* within a chromatin context. Such *in vitro* systems have been pivotal in understanding the relationship between chromatin structure and function in its role of regulating gene transcription.

In this work, we use a biochemical approach to understand the role of chromatin in transcription by using the HTLV-I promoter as a model system. An important element in defining the role of chromatin, and the regulatory proteins that interact with it, is the

creation of an *in vitro* system in which these interactions can be analysed. This chapter describes the development of such an *in vitro* chromatin and transcription system. We trial two methods commonly and successfully used in the literature to assemble purified DNA into chromatin. However, limitations of these systems for HTLV-I transcription necessitated the development of a novel *in vitro* chromatin assembly and transcription system to analyse the transcription factor DNA interactions within a chromatin context.

Numerous studies employing cell-free protein extracts have been used in the literature to reproduce *in vivo* factor-nucleosome interactions. For example, protein extracts prepared from *Xenopus laevis* oocytes have been particularly useful in assembling cloned genes into chromatin for *in vitro* transcription analysis (Glikin *et al.*, 1984, Tremethick *et al.*, 1990). Oocytes from *Xenopus* toads provide a rich source of nuclear proteins, including chromatin assembly components and transcription factors because such a store is required by rapidly dividing nuclei. These factors are able to mediate ATP facilitated and DNA replication independent assembly of chromatin that exhibits similar spacing of nucleosomes to that seen with native chromatin. The extract is obtained from the high-speed supernatant of *Xenopus* oocytes and is termed S-150. A similar extract derived from *Drosophila* embryos termed S-190 has also been extensively used for *in vitro* chromatin assembly (For example see Kamakaka *et al.*, 1993; Myall *et al.*, 1997; Kraus and Kadonaga, 1998). In this chapter, the S-150 extract from *Xenopus* oocytes was employed to assemble the HTLV-I and somatostatin promoters into chromatin and its usefulness in *in vitro* transcription assays was assessed.

A second method of chromatin assembly used in this chapter was the N1/N2(H3,H4) system (Tremethick *et al.*, 1990). In *Xenopus* oocytes, histones H3 and H4 are complexed with carrier proteins known as N1/N2 to produce a distinct complex N1/N2(H3,H4) with a sedimentation coefficient of 5S (Kleinschmidt and Seiter, 1988). In the oocyte, the assembly of nucleosomes is a two step process. Firstly, N1/N2 deposits H3/H4 onto the DNA to form a tetramer. secondly, H2A/H2B dimers combine with the tetramer to produce nucleosomes. In this *in vitro* study, histones H2A/H2B from *Xenopus* extracts were not used because of the presence of three different H2A variants (Shimamura *et al.*, 1988; Zucker and Worcel, 1990; Dilworth *et al.*, 1987) which could complicate the interpretation of the results. In this study, histones H2A and H2B purified from chicken red blood cells were used as this preparation consists of a single non-variant H2A and H2B species. This assembly system has been well characterised and assembles authentic chromatin with

different physiological nucleosomal repeat lengths when combined with ATP-dependent spacing fractions (Tremethick and Frommer, 1992; Tremethick and Hyman, 1996).

In this study, limitations of these chromatin assembly systems due to the presence of activators of HTLV-I transcription, necessitated the development of a method that assembles nucleosomes under physiological conditions with purified components. For this chromatin assembly system we utilised nucleosome assembly protein 1 (NAP-1) as a histone assembly factor. Histone assembly factors such as NAP-1, nucleoplasmin and N1/N2 function as histone transfer vehicles that facilitate the deposition of histones onto DNA in an ATP independent manner (Dilworth and Dingwall, 1988). NAP-1 derived from yeast (Fujii-Nakata *et al.*, 1992) was sub-cloned into a prokaryotic expression system and purified by nickel affinity chromatography. The recombinant NAP-1 was used to assemble purified H3/H4 tetramers and H2A/H2B dimers from chicken red blood cells. Therefore it was possible to assemble nucleosomes using pure components.

In addition to the development of a specific chromatin assembly system for HTLV-I, *in vitro* transcription systems were also optimised for each of the assembly systems used. It is desirable that the *in vitro* transcription system closely mimics the natural *in vivo* situation. Plasmids which incorporate the cloned somatostatin and HTLV-I promoters were used as templates for chromatin assembly. Plasmid templates allow the assembly of nucleosome arrays and are thought to more closely resemble the *in vivo* situation than linear DNA templates. As HTLV-I naturally infects and transforms T cells *in vivo* (Smith and Greene 1991a), transcription from the chromatin assembled templates utilised nuclear extract prepared from Jurkat T-cells. The nuclear extract provides RNA polymerase II and the general transcriptional machinery for *in vitro* transcription. Although chromatin may play a major role in *in vivo* HTLV-I transcription, transcription from HTLV-I has not been analysed in a chromatin environment and therefore the effect of chromatin assembly on HTLV-I transcription is unknown. As in the previous chapters, the cellular CRE element from the somatostatin gene is used as a control for HTLV-I chromatin assembly and transcription.

5.2 Results

5.2.1 The development of an in vitro *transcription system*

5.2.1.1 Transcription templates

Plasmid DNA for use as the template for chromatin assembly is required to be less than 4 kb to ensure efficient assembly (Tremethick, 1999). A 700 bp fragment from the 3' HTLV-I promoter was sub-cloned into pBluescript (pBS) to create a suitable template for chromatin assembly (section 2.5.1). A map depicting the major regulatory elements of the HTLV-I and somatostatin promoter templates is shown in figure 5.1. Importantly, the templates used for chromatin assembly and *in vitro* transcription are derived from the naturally occurring HTLV-I sequence. The major late promoter from adenovirus (pMLP) was used as a control for *in vitro* transcription. This promoter has been widely used for *in vitro* transcription and is well characterised in the literature (Tremethick, 1994).

5.2.1.2 *In vitro* transcription from naked DNA templates

In preparation for transcription from chromatin assembled templates, *in vitro* transcription was performed on naked HTLV-I, somatostatin and pMLP DNA (Figure 5.2) as described in the methods (section 2.5). The site of HTLV-I transcription initiation in *in vitro* transcription assays has previously been shown to be the same as the site used *in vivo* (Piras *et al.*, 1994). For the *in vitro* transcriptions carried out in this study, the RNA transcripts were detected by primer extension (section 2.5.5). The positions of the primers for the HTLV-I and somatostatin templates are shown in figure 5.1 Figure 5.2 shows a titration of DNA template for HTLV-I (panel A), pMLP (panel B) and somatostatin (panel C). The level of basal transcription from HTLV-I templates is considerably less than transcription levels generated using the same concentration of somatostatin template (compare panels A and C, lanes 1, 2 and 3). Because of the low levels of basal transcription, the relative concentrations of DNA and Jurkat transcription extract, $MgCl_2$ concentration and the annealing temperature were each optimised to generate maximum transcription from the naked DNA templates (results not shown). Transcription was not detected when the RNA pol II inhibitor α-amanitin was included in the reaction with the HTLV-I (panel A lanes 4, 5 and 6) or the pMLP (panel B, lane 4) templates indicating that the transcription is specific for RNA pol II.

A

-354
5'-TGACAATGACCATGAGCCCCAAATATCCCCCGGGGGCTTAGAGCCTCTCAGTGAAAAACA

-294
TTTCCGTGAAACAGAAGTCTGAGAAGGTCAGGGCCCAGAATAAGGCTCTGACGTCTCCCC
TRE-3 (dLTR)

-234
CCGGAGGACAGCTCAGCACCAGCTCAGGCTAGGCCCTGACGTGTCCCCCTAAAGACAAAT
TRE-2 (mLTR)

-174
CATAAGCTCAGACCTCCGGGAAGCCACCGGGAACCACCCATTTCCTCCCCATGTTTGTCA

-114
AGCCGTCCTCAGGCGTTGACGACAACCCCTCACCTCAAAAAACTTTTCATGGCACGCATA
TRE-1(pLTR)

-54
CGGCTCAATAAAATAACAGGAGTCTATAAAAGCGTGGGGACAGTTCAGGAGGGGGCTCGC
+1
TATA-Box

+8
ATCTCTCCTTCACGCGCCCGCCGCCTTACCTGAGGCCGCCATCCACGCCGGTTGAGTCGC
3'-GGTGCGGCCAACTCAGCG

+68
GTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTACGTCCGCCGTCTAGGTAAGTTTA
CAA-5'

+128
GAGCTCAGGTCGAGACCGGGCCTTTGTCCGGCGCTCCCTTGGAGCCTACCTAGACTCAGC

+188
CGGCTCTCCACGCTTTGCCTGACCCTGCTTGCTCAACTCTACGTCTTTGTTTCGTTTTCT

+248
GTTCTGCGCCGTTACAGAT-3'

B

-71
5'-AGATCTGGGGGCGCCTCCTTGGCTGACGTCAGAGAGAGAGTTTAAAAAGGGGAGACCGT
+1
CRE
TATA-BOX

-12
GGAGAGCTCGATAGCGGCTGAAGGAGACGCTACTGGAGTCGTCTCTGCTGCCTGCGGAC

+48
CTGCGTCTAGAGTAGACATGCAGCCCCAAGCTTGGCGAGATTTTCAGGAGCTAAGGAAG
pSynCAT
CAT
3'-TTCGAACCGCTCTAAAAGTCCT-5'
CTAAAATG-3'

Figure 5.1: Sequence of the HTLV-1 and somatostatin promoter regions used as templates for *in vitro* transcription. A. Sequence of the 700 bp fragment (-354 to + 266) from the HTLV-I 3' LTR. The position of the primers used for primer extension analysis of the transcription products is shown in red. The sequence is numbered relative to the start site of transcription (+1). The TATA-box, and the Tax responsive elements are indicated. **B.** Sequence of the somatostatin promoter (-71 to + 58) used as a template for *in vitro* transcription. Sequence directly from the somatostatin gene is shown in dark type and sequence from the CAT reporter gene (and sequence from the pSynCAT vector) is shown in light type. The position of the primer used for primer extension analysis is shown in red. Sequence is numbered relative to the start site of transcription and the cAMP responsive element (CRE) and the TATA-box is indicated

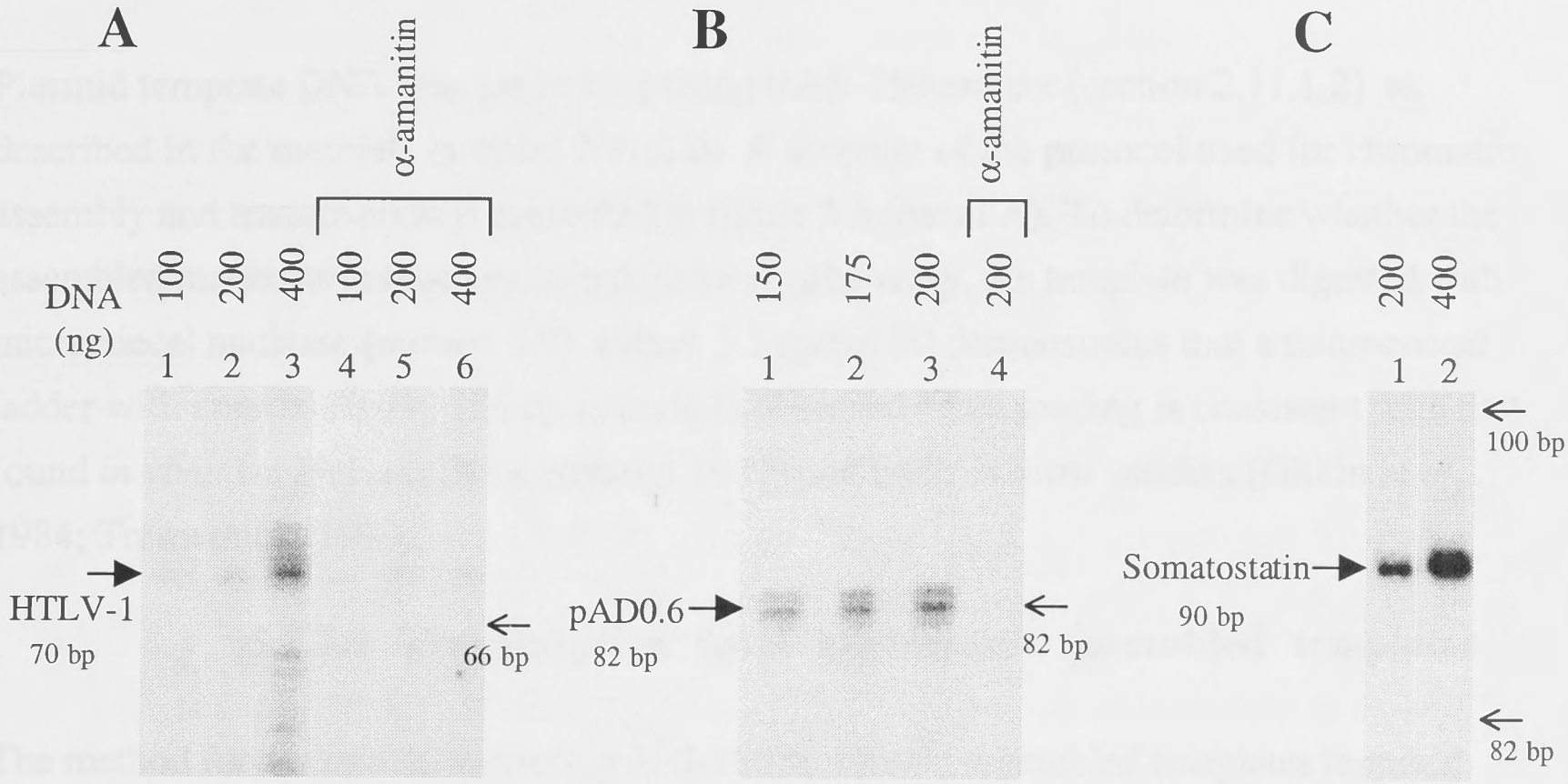


Figure 5.2: *In vitro* transcription from naked DNA templates. Naked DNA templates were transcribed *in vitro* in the presence or absence of the RNA pol II inhibitor α-amanitin (final concentration 1.5 μg/ml). Transcription products were visualised by resolving the products of primer extension analysis on a 8%/7 M urea sequencing gel. **A.** *In vitro* transcription from naked HTLV-I templates. Lanes 1, 2 and 3: 100, 200 and 400 ng of HTLV-I template respectively; lanes 4, 5 and 6: α-amanitin with 100, 200 and 400 ng of template respectively. **B.** *In vitro* transcription from the adenovirus major late promoter (pAD0.6). Lanes 1, 2 and 3: 150, 175 and 200 ng DNA template; lane 4: 200 ng DNA template with α-amanitin. **C.** *In vitro* transcription from somatostatin templates. Lane 1 and 2: 200 and 400 ng of template respectively. The position of the transcription products are indicated by an arrow and the positions of the molecular weight markers (bp) are shown on the right of each figure.

5.2.2 Transcription from nucleosome assembled HTLV-I and somatostatin templates using the Xenopus S-150 chromatin assembly extract

5.2.2.1 Nucleosome assembly

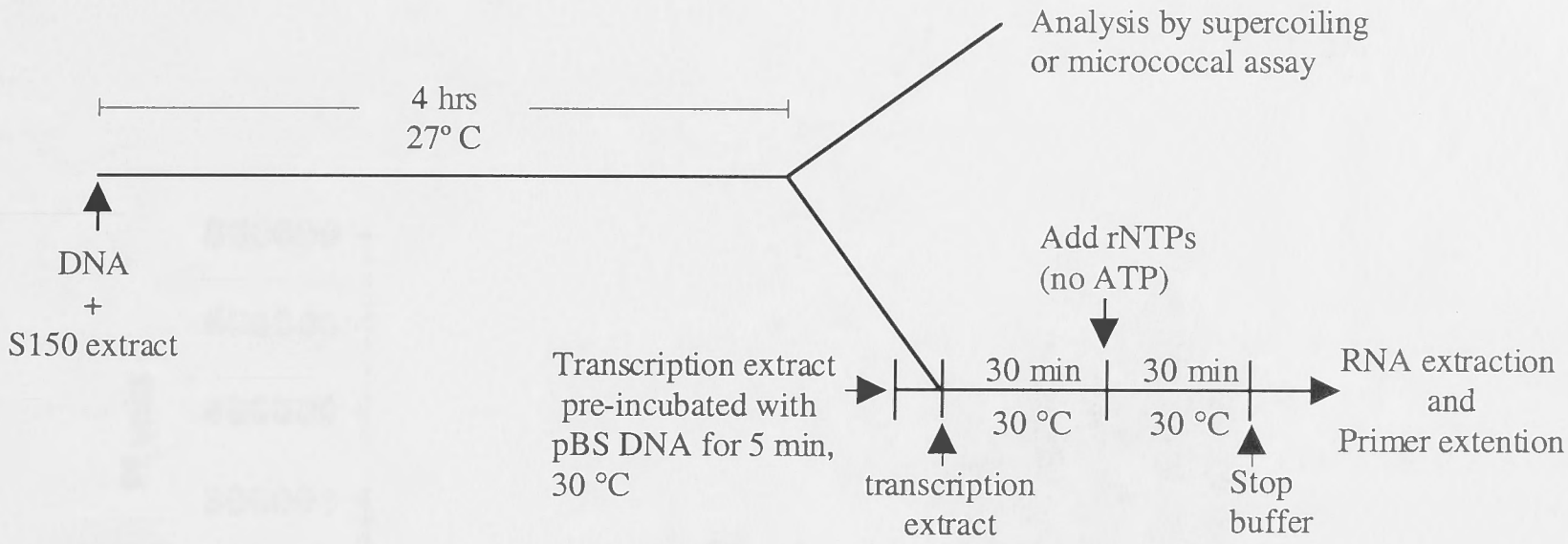
Plasmid template DNA was assembled using the S-150 extract (section 2.11.1.2) as described in the methods (section 2.11.2.2). A diagram of the protocol used for chromatin assembly and transcription is presented in figure 5.3 (panel A). To determine whether the assembled nucleosomes are organised into a regular array, the template was digested with micrococcal nuclease (section 2.9). Figure 5.3 (panel B) demonstrates that a micrococcal ladder with approximately 185 bp spacing is observed. This spacing is consistent with that found *in vivo* for HeLa cells (Kornberg, 1977) and other *in vitro* studies (Glikin *et al*, 1984; Tremethick, 1999).

5.2.2.1 Transcription from nucleosome assembled templates

The method for *in vitro* transcription of S-150 chromatin assembled templates required considerable optimisation to accommodate differences in conditions between naked DNA and chromatin assembled DNA. For example, chromatin assembly required the presence of 3 mM ATP, however, this concentration of ATP inhibited transcription from HTLV-I templates (results not shown). Moreover, to maintain the appropriate salt concentration for assembly and transcription, the maximum amount of DNA transcribable was 300 ng. Basal transcription from HTLV-I templates is considerably less than commonly used templates such as the adenovirus major late promoter and the somatostatin gene (section 5.2.1.2, figure 5.2). Therefore, under the conditions required for nucleosome assembly, basal HTLV-I transcription was undetectable. To overcome this problem, the transcription extract was pre-incubated with an empirically determined concentration of pBS DNA for 5 minutes before addition to the transcription reaction (figure 5.3, panel A). This DNA is thought to bind non-specific inhibitors of transcription present in the nuclear extract and its addition to the transcription extract resulted the detection of HTLV-I transcripts.

To assess the viability of the S-150 chromatin assembly system for use in *in vitro* transcription, the assembled templates were transcribed *in vitro* according to the methods (section 2.5). Figure 5.4 shows a titration of nuclear extract into 300 ng of mock assembled (lanes 1-3 and 7-9) or assembled (lanes 4-6 and 10-12) HTLV-I (lanes 1-6) and somatostatin (lanes 7-12) templates. For both the somatostatin and HTLV-I templates,

A



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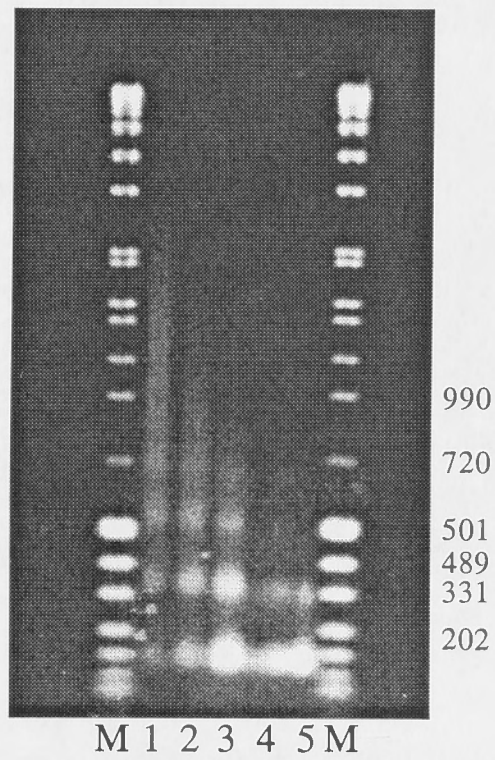


Figure 5.3: Chromatin assembly using the s150 *Xenopus* oocyte protein extract. A. A schematic diagram of the protocol used for assembly of plasmid DNA templates using the S-150 extract. **B.** Micrococcal nuclease digestion ladder of the HTLV-I plasmid assembled into chromatin by the S-150 chromatin assembly system. Lanes 1-5 were digested with 50 U/μl micrococcal nuclease for 1, 2, 4, 8 and 16 minutes respectively. Molecular size markers (bp) are shown at the right of the figure.

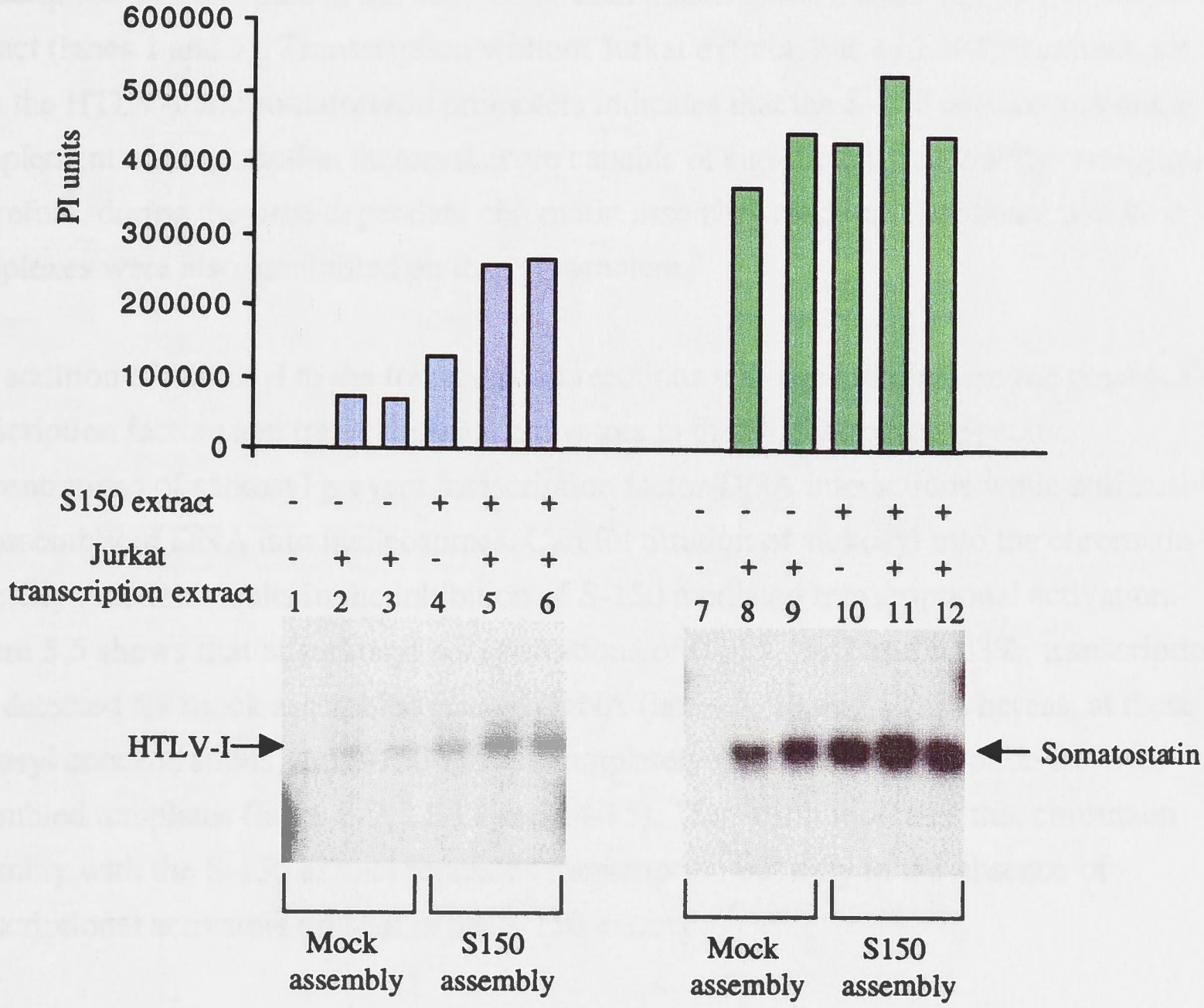


Figure 5.4: Chromatin assembly using the S-150 *Xenopus* oocyte extract does not repress basal transcription. 300 ng of HTLV-I (lanes 1-6) or somatostatin (lanes 7-12) template DNA were either mock assembled (naked DNA) or assembled with the S-150 *Xenopus* oocyte extract. The templates were transcribed *in vitro* with increasing concentrations of Jurkat transcription extract. Lanes 1-3 and 7-9: Mock assembled template with 0, 75 and 100 μg nuclear extract respectively; lanes 4-6 and 10-12: Assembled template with 0, 75 and 100 μg nuclear extract. The transcription products were quantitated using a phosphoimager and image gauge software. The quantitation data is presented as a graph above the figure and the units of quantitation are phosphoimage (PI) units.

transcription from assembled DNA was greater that that detected for mock-assembled (naked) DNA (compare lanes 4-5 with 1-3 and lanes 10-12 with 7-9). This result was somewhat surprising as in general, chromatin assembly has been shown to repress transcription. However, assembled templates showed less, but significant levels of transcription in the absence of Jurkat transcription extract (lanes 4 and 10), whereas, no transcription was detected in the absence of both transcription extract and S-150 assembly extract (lanes 1 and 7). Transcription without Jurkat extract, but with S-150 extract, for both the HTLV-I and somatostatin promoters indicates that the S-150 extract contains a complement of transcription factors that are capable of significantly activating transcription. Therefore, during the time dependent chromatin assembly reaction, functional initiation complexes were also assembled on these promoters.

The addition of sarkosyl to the transcription reactions was used to confirm the presence of transcription factors and transcriptional activators in the S-150 extract. Specific concentrations of sarkosyl prevent transcription factor-DNA interactions while still enabling the assembly of DNA into nucleosomes. Careful titration of sarkosyl into the chromatin assembly reaction results in the inhibition of S-150 mediated transcriptional activation. Figure 5.5 shows that at sarkosyl concentrations of 0.015, 0.02 and 0.03%, transcription was detected for mock assembled (naked) DNA (lanes 7, 10 and 13). Whereas, at these sarkosyl concentrations, the S-150 extract completely repressed transcription from the assembled templates (lanes 8-9, 11-12 and 14-15). This result indicates that chromatin assembly with the S-150 extract represses transcription but only in the absence of transcriptional activators present in the S-150 extract.

The overall aim of this work is to examine the effect of the HTLV-I specific transcriptional activators Tax, CREB and CBP in a repressed chromatin environment. Since assembly with S-150 does not result in transcriptional repression, in contrast to studies using the *Xenopus* 5S RNA gene (Shimamura *et al.*, 1988) the S-150 system of chromatin assembly was not subsequently used.

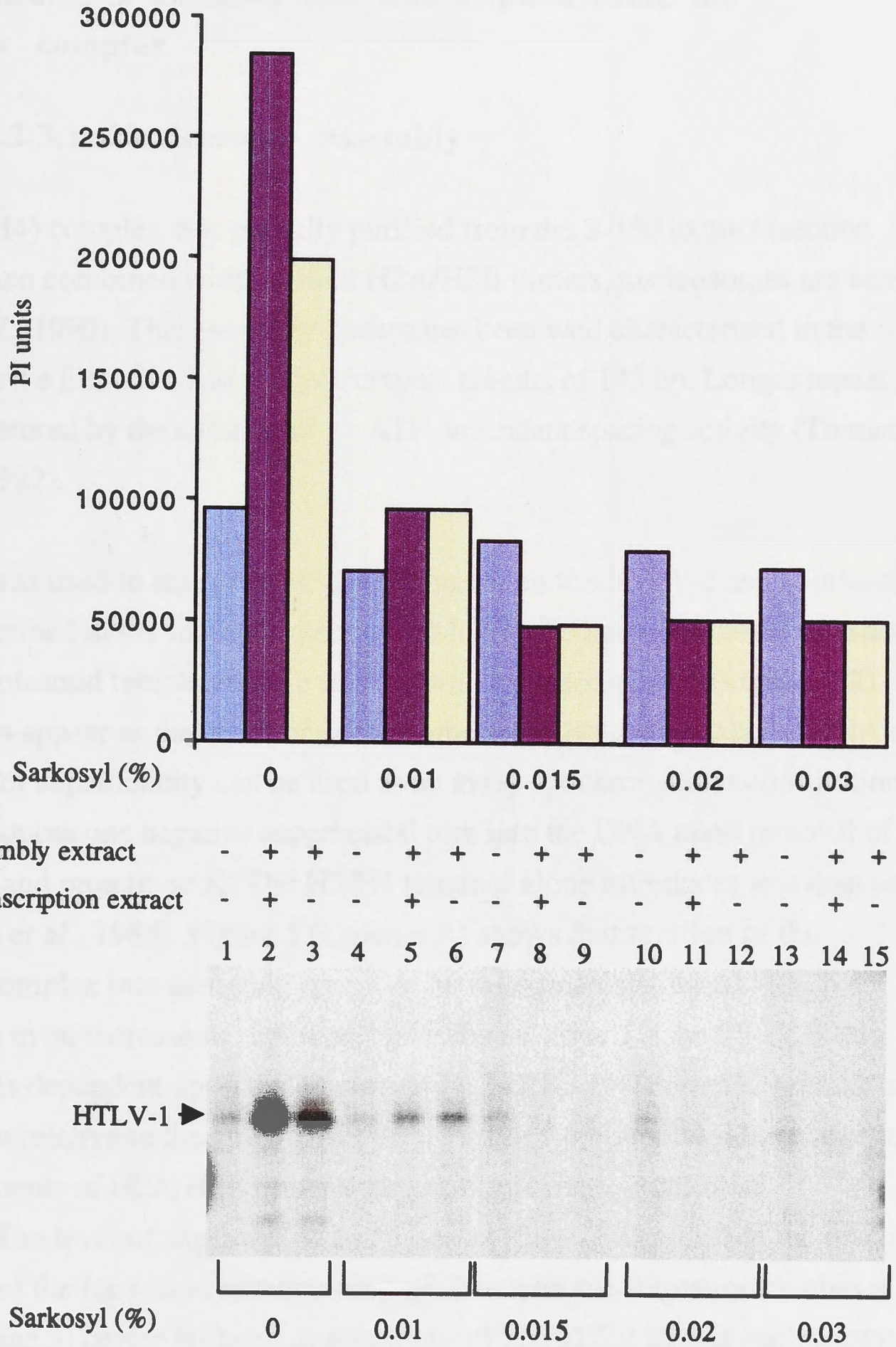


Figure 5.5: Chromatin assembly in the presence of sarkosyl represses basal transcription. HTLV-I template DNA was assembled with the S-150 extract in the presence of sarkosyl. Sarkosyl concentrations are shown below the figure. Assembled (lanes 2-3, 5-6, 8-9, 11-12 and 14-15) or mock assembled (naked DNA) (lanes 1, 4, 7, 10 and 13) template was transcribed *in vitro* in the presence (lanes 2, 5, 8, 11 and 14) or absence (lanes 3, 6, 9, 12 and 15) of Jurkat transcription extract (100 μg). The products from the *in vitro* transcription reaction were resolved on a sequencing gel and exposed to a phosphoimage screen. The transcription products were quantitated using image gauge software and the units of quantitation are phosphoimage (PI) units. The quantitation results are shown above the figure.

5.2.3 Chromatin assembly and transcription using the N1/N2(H3,H4) complex

5.2.3.1 Nucleosome assembly

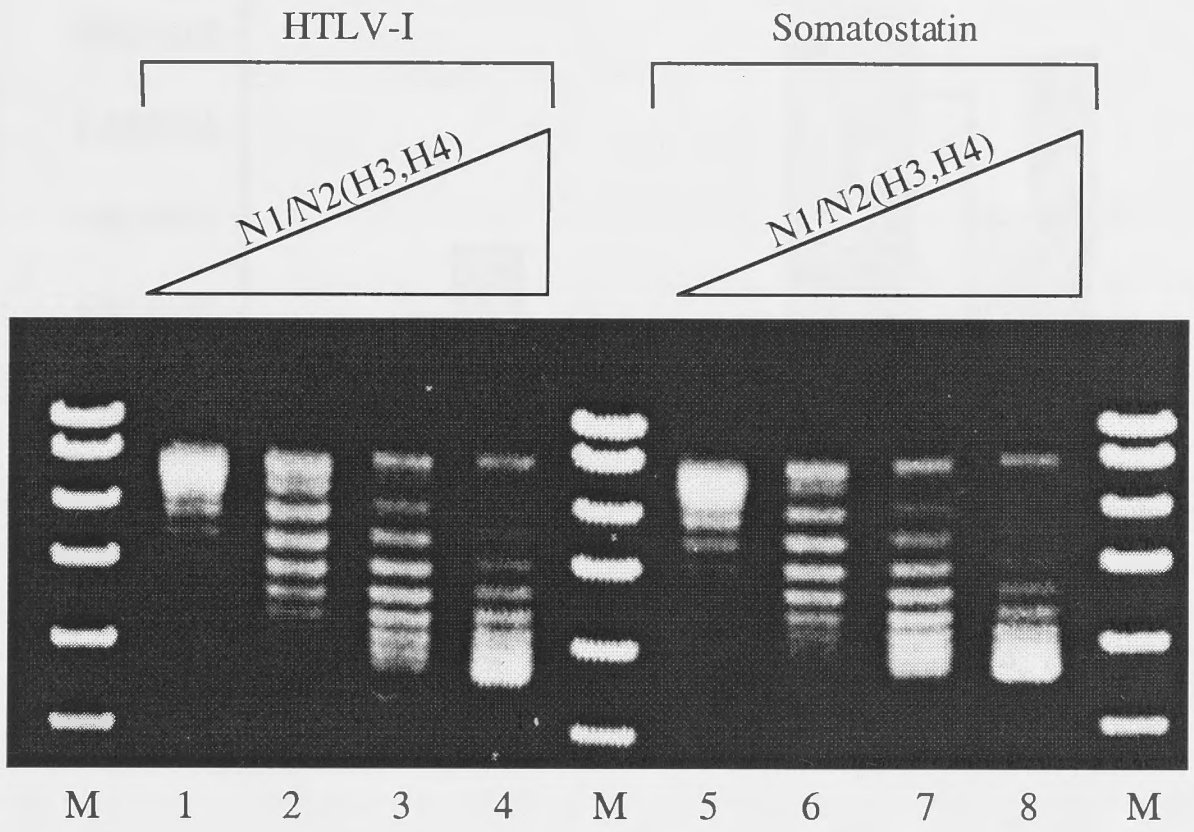
The N1/N2(H3,H4) complex was partially purified from the S-150 extract (section 2.11.1.3) and when combined with purified H2A/H2B dimers, nucleosomes are assembled (Tremethick *et al.*, 1990). This assembly system has been well characterised in the laboratory and in the literature and produces repeat lengths of 145 bp. Longer repeat lengths can be restored by the addition of an ATP dependant spacing activity (Tremethick and Frommer, 1992).

N1/N2(H3,H4) was used to assemble H3/H4 tetramers on the HTLV-I and somatostatin templates as described in the methods (section 2.11.2.3). To assess the level of assembly of the DNA, the plasmid templates were relaxed with topoisomerase I (section 2.8). Superhelical turns appear as the result of nucleosome assembly on the relaxed DNA and this introduction of superhelicity can be used as an assay for chromatin reconstitution. One nucleosome introduces one negative superhelical turn into the DNA upon removal of histones by SDS and proteinase K. The H3/H4 tetramer alone introduces less than one supercoil (Glikin *et al.*, 1984). Figure 5.6, (panel A) shows that titration of the N1/N2(H3,H4) complex into assembly reactions on the somatostatin and HTLV-I promoters results in an increase in supercoiling (compare lanes 1-4, and 5-8). Therefore the tetramer density is dependent upon the amount of N1/N2(H3,H4) complex present in the assembly reaction relative to the amount of DNA. Figure 5.6, (panel B) shows the titration of increasing amounts of H2A/H2B dimer with a constant concentration of N1/N2(H3,H4). The level of supercoiling increases as H2A/2B is added to the reaction and is representative of the formation of a nucleosome. Nucleosome formation reaches a optimum level (lane 5) before higher concentrations of H2A/H2B inhibit nucleosome formation.

5.2.3.2 Transcription from nucleosome assembled templates

To assess the effect of increasing nucleosome density on the transcription of HTLV-I and somatostatin promoters, increasing amounts of N1/N2(H3,H4) complex were added to the assembly reactions with a fixed level of recombinant H2A/H2B. Figure 5.7 (panel A) shows that titration of N1/N2(H3,H4) into the somatostatin assembly reaction resulted in repression of transcription (compare lanes 7-10). This repression was due to

A



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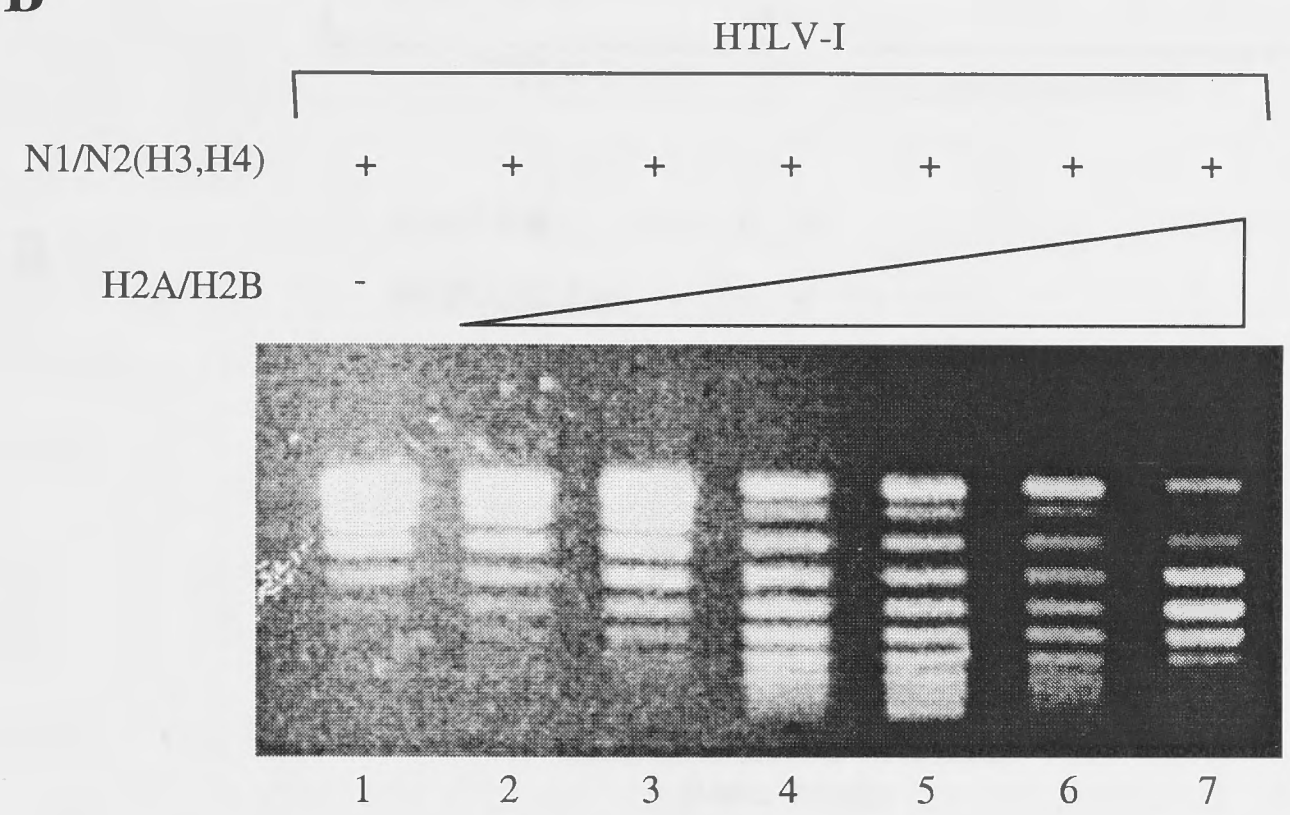


Figure 5.6: Nucleosome assembly using the N1/N2(H3,H4) complex.
Supercoiling analysis of assembly using components of the N1/N2(H3,H4) assembly system in the presence of 2 U topoisomerase I. **A.** Increasing amounts of N1/N2(H3,H4) complex was incubated with HTLV-I (lanes 1-4) or somatostatin (lanes 5-8) template DNA. Lanes 1-4 and 5-9: 0, 10, 15 and 20 µl of N1/N2(H3,H4) complex respectively. **B.** Supercoiling analysis of HTLV-I templates assembled with increasing amounts of purified H2A/H2B dimer from long chicken chromatin with a constant concentration (10 µl) of N1/N2(H3,H4). Lane 1-7: 0, 0.5, 1, 3, 5, 7.5 and 10 µl H2A/H2B respectively.

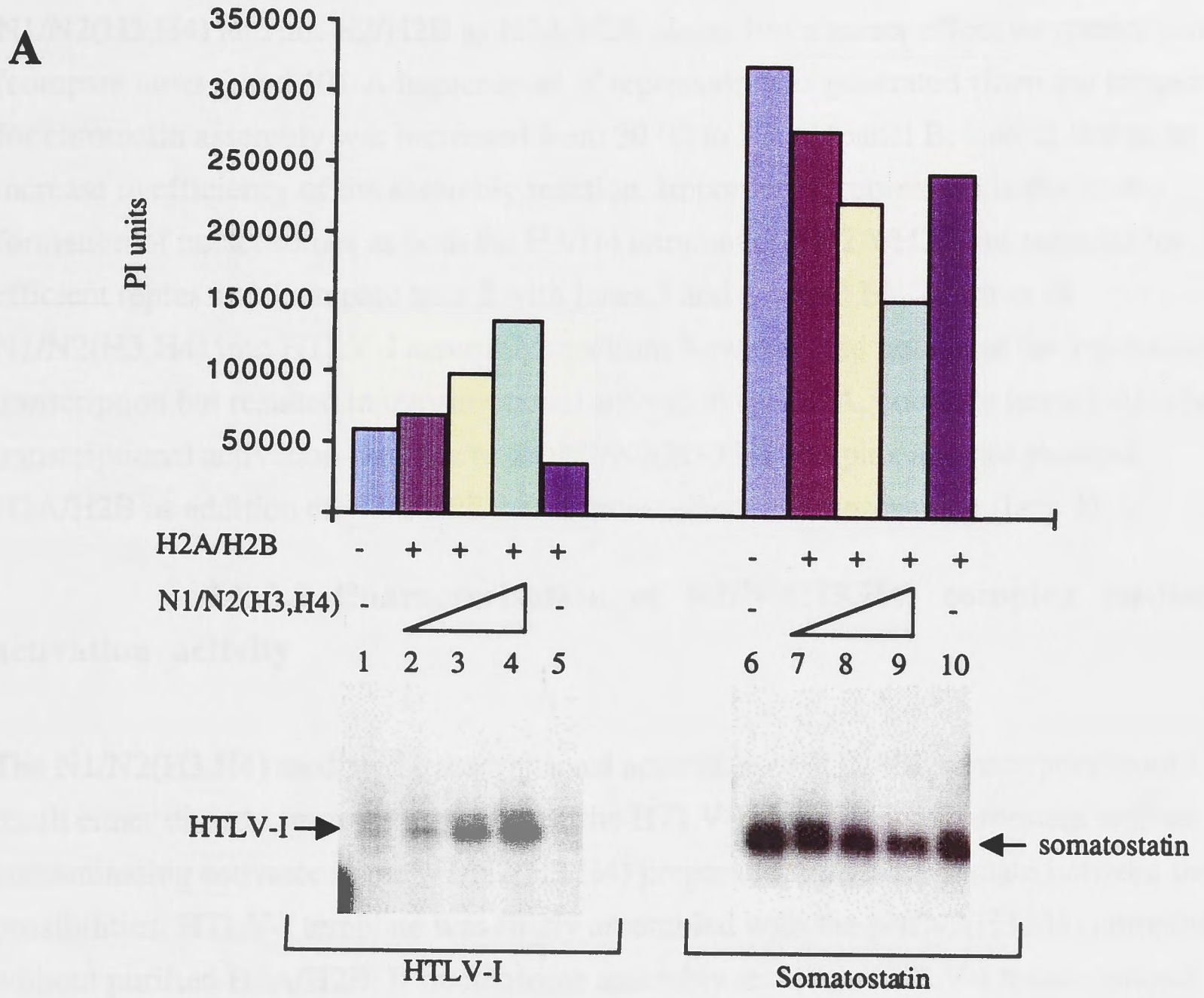


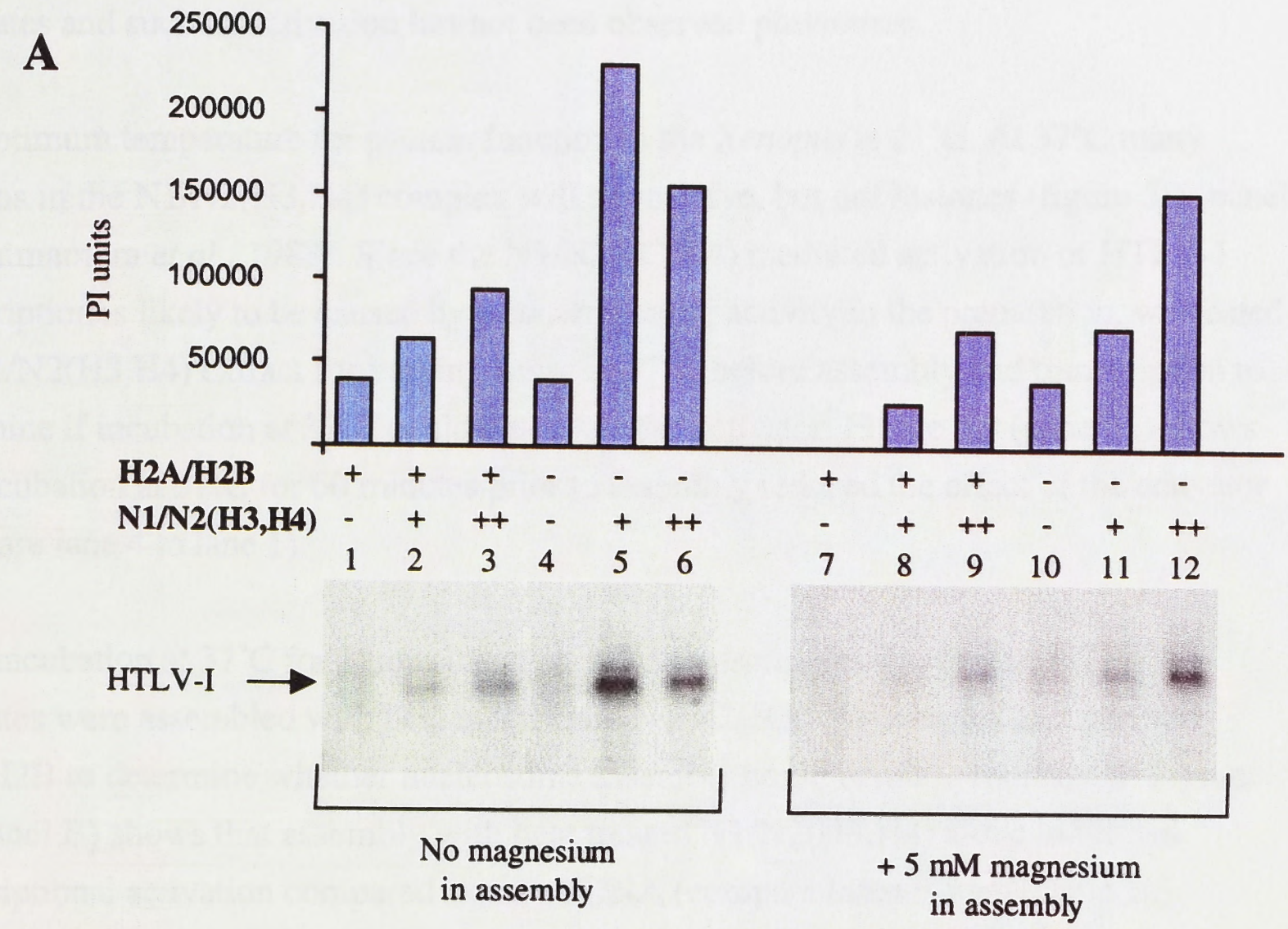
Figure 5.7 Nucleosome assembly of HTLV-I template using the N1/N2(H3/H4) system does not repress basal transcription. A. 300 ng of HTLV-I (lanes 1-5) or somatostatin (lanes 6-10) template DNA was assembled using the components of the N1/N2(H3/H4) assembly system. Lanes 1 and 6: Mock assembled (naked DNA); lanes 2 and 7: 10 µl N1/N2(H3,H4) and 5 µl H2A/H2B; lane 3 and 8: 15 µl N1/N2(H3,H4) and 5 µl H2A/H2B; lanes 4 and 19; 20 µl of N1/N2(H3,H4) and 5 µl of H2A/H2B; lanes 5 and 10 contain 5 µl H2A/H2B. The templates were transcribed *in vitro*, resolved on a sequencing gel and exposed to a phosphoimage screen. The transcription products were quantitated using image gauge software and the units of quantitation are phosphoimage (PI) units. The quantitation results are shown above the figure. **B.** *In vitro* transcription of somatostatin template incubated with components of the N1/N2(H3,H4) assembly system 37°C for 4 hours. Lane 1: Mock assembled (naked DNA); lane 2: 20 µl of N1/N2(H3,H4) and 5µl of H2A/H2B (fully assembled); lane 3: 5 µl H2A/H2B; lane 4: 20 µl N1/N2(H3,H4).

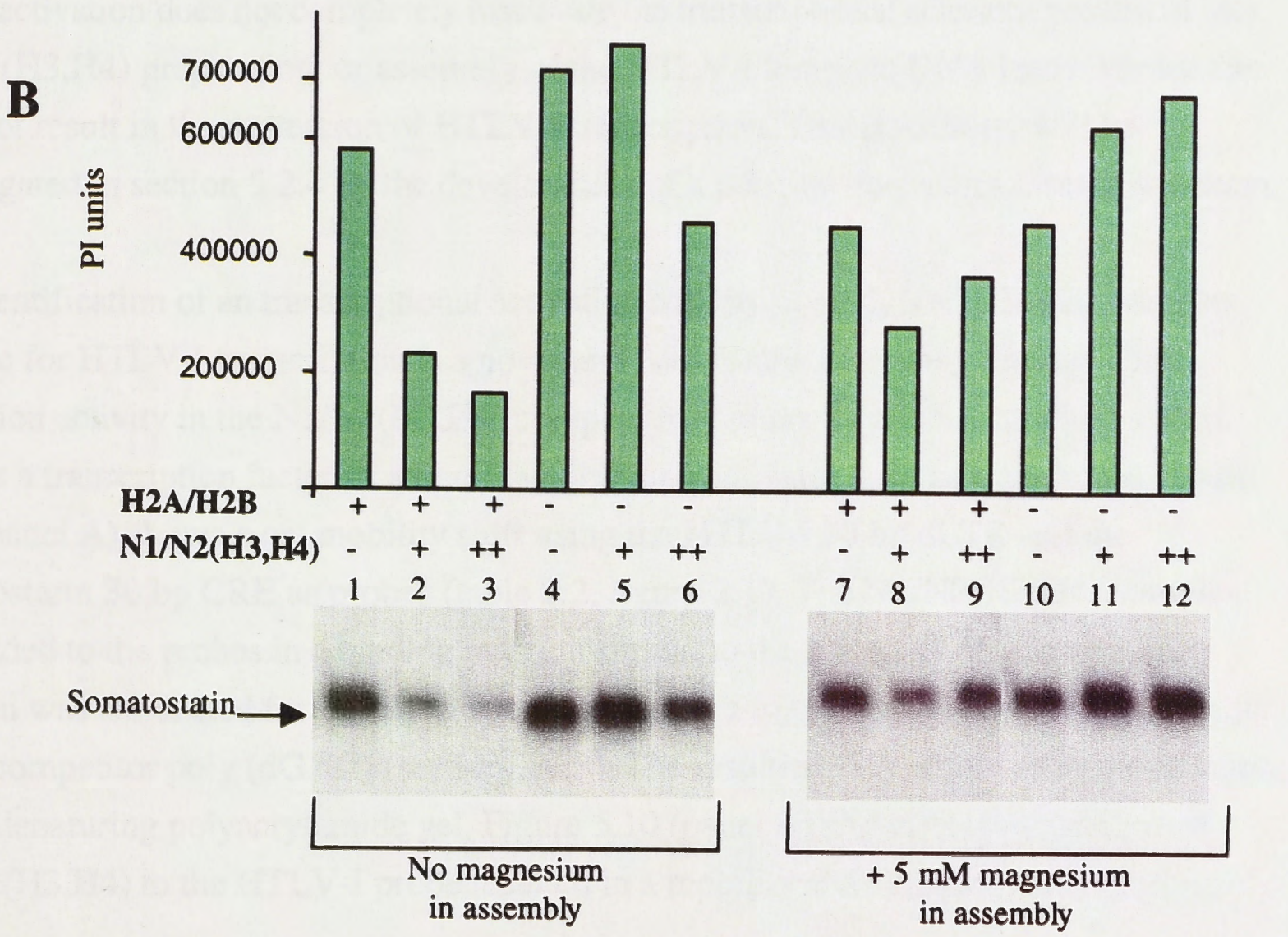
N1/N2(H3,H4) and not H2/H2B as H2A/H2B alone, has a minor effect on transcription (compare lanes 6 and 10). A higher level of repression was generated when the temperature for chromatin assembly was increased from 30 ˚C to 37 ˚C (panel B, lane 2) due to an increase in efficiency of the assembly reaction. Importantly, repression is due to the formation of nucleosomes as both the H3/H4 tetramer and H2A/H2B are required for efficient repression (compare lane 2 with lanes 3 and 4, panel B). Titration of N1/N2(H3,H4) into HTLV-I assembly reactions however, did not cause the repression of transcription but resulted in transcriptional activation (panel A, compare lanes 1-4). The transcriptional activation was due to the N1/N2(H3,H4) complex and not purified H2A/H2B as addition of H2A/H2B had a minor effect on transcription (lane 5).

5.2.3.3 Characterisation of N1/N2(H3,H4) complex mediated activation activity

The N1/N2(H3,H4) mediated transcriptional activation of HTLV-I transcription could result either directly from the assembly of the HTLV-I template into chromatin or from a contaminating activator in the N1/N2(H3,H4) preparation. To discriminate between these possibilities, HTLV-I template was firstly assembled with the N1/N2(H3,H4) complex without purified H2A/H2B. If nucleosome assembly results in HTLV-I transcriptional activation, assembly using H3/H4 tetramers should not result in a sufficient level of compaction to cause this effect. Figure 5.8 (panel A) shows that assembly of HTLV-I with H3/H4 alone results in transcriptional activation (lanes 4, 5 and 6). By contrast, assembly of the somatostatin template with H3/H4 alone has minimal effect on the transcription level (panel B, lane 4, 5 and 6) indicating that assembly of H3/H4 tetramers is insufficient to cause repression. Furthermore, 5 mM $MgCl_2$ has been shown to inhibit nucleosome assembly by inhibiting the formation of the H3/H4 tetramers (Tremethick *et al.*, 1990). Therefore, if nucleosome assembly directly results in HTLV-I transcription activation, assembly in the presence of 5 mM $MgCl_2$ should negate this activation. Figure 5.8 (panel A) shows that assembly of HTLV-I templates in the presence of $MgCl_2$ results in transcriptional activation (lanes 7-9). However, assembly of the control template, somatostatin, in the presence of $MgCl_2$, prevents assembly as evidenced by the reduced levels of repression (panel B lanes 7-9). These results suggest that nucleosome assembly is not directly responsible for the transcriptional activation of the HTLV-I template and imply that a contaminating activity present in the N1/N2(H3,H4) preparation is responsible for the HTLV-I specific transcriptional activation. It is worth noting that this laboratory has

Figure 5.8: Characterisation of N1/N2(H3,H4) complex mediated activation activity. A. *In vitro* transcription from HTLV-I DNA assembled with increasing amounts of N1/N2(H3,H4) complex in the absence (lanes 1-6) or presence (lanes 7-12) of 5 mM $MgCl_2$. Lanes 1-3 and 7-9: 5 µl H2A/H2B with either 0, 15 and 20 µl of N1/N2(H3,H4) complex respectively; lanes 4-6 and 10-12: Either 0, 15 or 20 µl of N1/N2(H3,H4) complex respectively**. B** *In vitro* transcription from somatostatin templates assembled with increasing amounts of N1/N2(H3,H4) complex in the absence (lanes 1-6) or presence (lanes 7-12) of 5 mM $MgCl_2$. Lanes as for panel A. Transcription products were resolved on a sequencing gel and exposed to a phosphoimage screen. The transcription products were quantitated using image gauge software and the units of quantitation are phosphoimage (PI) units. The quantitation results are shown above the figure.


A
250000
200000
150000
100000
50000
PI units
H2A/H2B + + + - - - + + + - - -
N1/N2(H3,H4) - + ++ - + ++ - + ++ - + ++
1 2 3 4 5 6 7 8 9 10 11 12
HTLV-I
No magnesium in assembly
+ 5 mM magnesium in assembly


B
700000
600000
400000
200000
PI units
H2A/H2B + + + - - - + + + - - -
N1/N2(H3,H4) - + ++ - + ++ - + ++ - + ++
1 2 3 4 5 6 7 8 9 10 11 12
Somatostatin
No magnesium in assembly
+ 5 mM magnesium in assembly

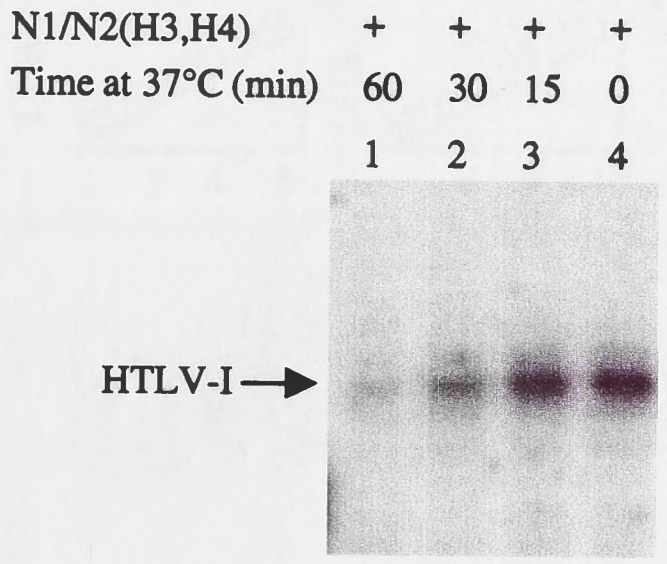
extensively used this system to repress the transcription of a large number of different templates and such an activation has not been observed previously.

The optimum temperature for protein function in the *Xenopus* is 21°C. At 37°C many proteins in the N1/N2(H3,H4) complex will be inactive, but not histones (figure 5.7, panel B) (Shimamura *et al.*, 1988). Since the N1/N2(H3,H4) mediated activation of HTLV-I transcription is likely to be caused by a contaminating activity in the preparation, we heated the N1/N2(H3,H4) extract for varying times at 37°C before assembly and transcription to determine if incubation at 37°C could inactivate the activator. Figure 5.9 (panel A) shows that incubation at 37°C for 60 minutes prior to assembly reduced the effect of the activator (compare lane 4 to lane 1).

Since incubation at 37°C for 1 hour inactivates the transcriptional activator, HTLV-I templates were assembled with heat inactivated N1/N2(H3,H4) complex and purified H2A/H2B to determine whether nucleosome assembly could result in repression. Figure 5.9 (panel B) shows that assembly with heat treated N1/N2(H3,H4) alone exhibited transcriptional activation compared to naked DNA (compare lanes 5-8 with lane 10). Nucleosome assembly with the heat-treated N1/N2(H3,H4) and H2A/H2B resulted in lower levels of transcription. However the transcription from the assembled templates was not significantly lower than naked DNA (compare lanes 1-4 with lane 10). Therefore, either heat inactivation does not completely inactivate the transcriptional activator present in the N1/N2(H3,H4) preparation, or assembly of the HTLV-I template DNA into nucleosomes does not result in the repression of HTLV-I transcription. This possibility will be investigated in section 5.2.4 by the development of a purified chromatin assembly system.

The identification of an transcriptional activation activity in the N1/N2(H3,H4) complex specific for HTLV-I transcription is a novel and potentially interesting finding. This activation activity in the N1/N2(H3,H4) complex may either be a DNA binding protein such as a transcription factor or a non-DNA-binding activator such as a co-factor. Figure 5.10 (panel A) shows a gel mobility shift using the HTLV-I 30 bp dLTR and the somatostatin 30 bp CRE as probes (table 2.2, figure 2.1). The N1/N2(H3,H4) complex was added to the probes in a binding reaction similar to the assembly reaction but the reaction was conducted for 30 min at room temperature in the presence of the non-specific DNA competitor poly (dG.dC) (section 2.4.3). The resulting complexes were resolved on a non-denaturing polyacrylamide gel. Figure 5.10 (panel A) shows that the binding of N1/N2(H3,H4) to the HTLV-I probe resulted in a mobility shift indicating the binding of a

A



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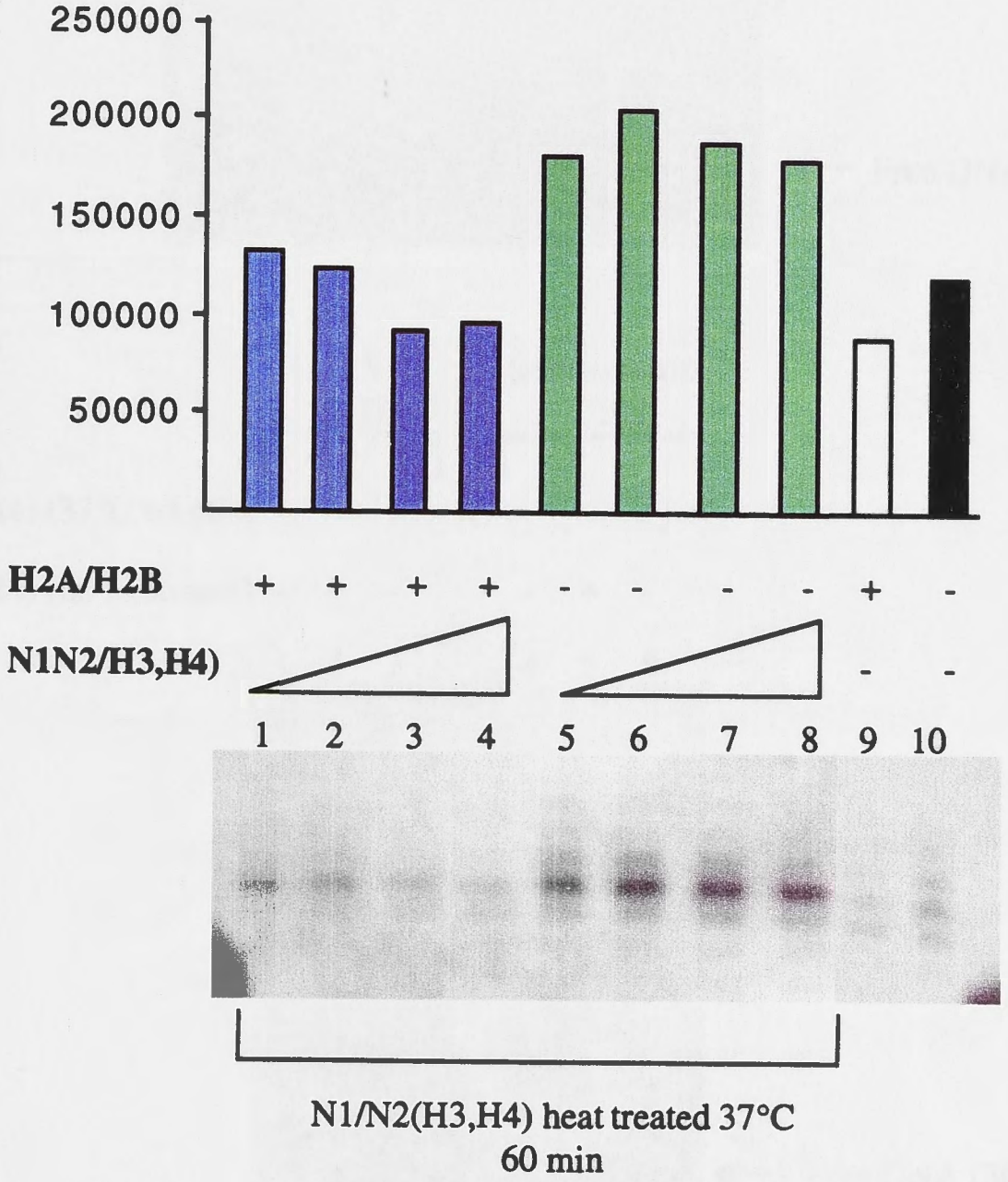


Figure 5.9: Heat inactivated N1/N2(H3,H4) complex does not repress basal transcription from HTLV-I templates. A. 20 µl of N1/N2(H3,H4) extract was incubated at 37°C for the times indicated above the figure. The heat treated complexes were used to assemble HTLV-I template DNA and templates were analysed by *in vitro* transcription. **B.** N1/N2(H3,H4) was incubated at 37°C for 60 min and used in combination with H2A/H2B to assemble HTLV-I template DNA. Lanes 1-4: 5 µl H2A/H2B with either 10, 15, 20 or 25 µl of N1/N2(H3,H4); lanes 5-8; Either 10, 15, 20 or 25 µl of N1/N2(H3,H4); lane 9: 5 µl of H2A/H2B; lane 10: Mock assembled (naked DNA). The transcription products were quantitated using image gauge software and the units of quantitation are phosphoimage (PI) units. The quantitation results are shown above the figure.

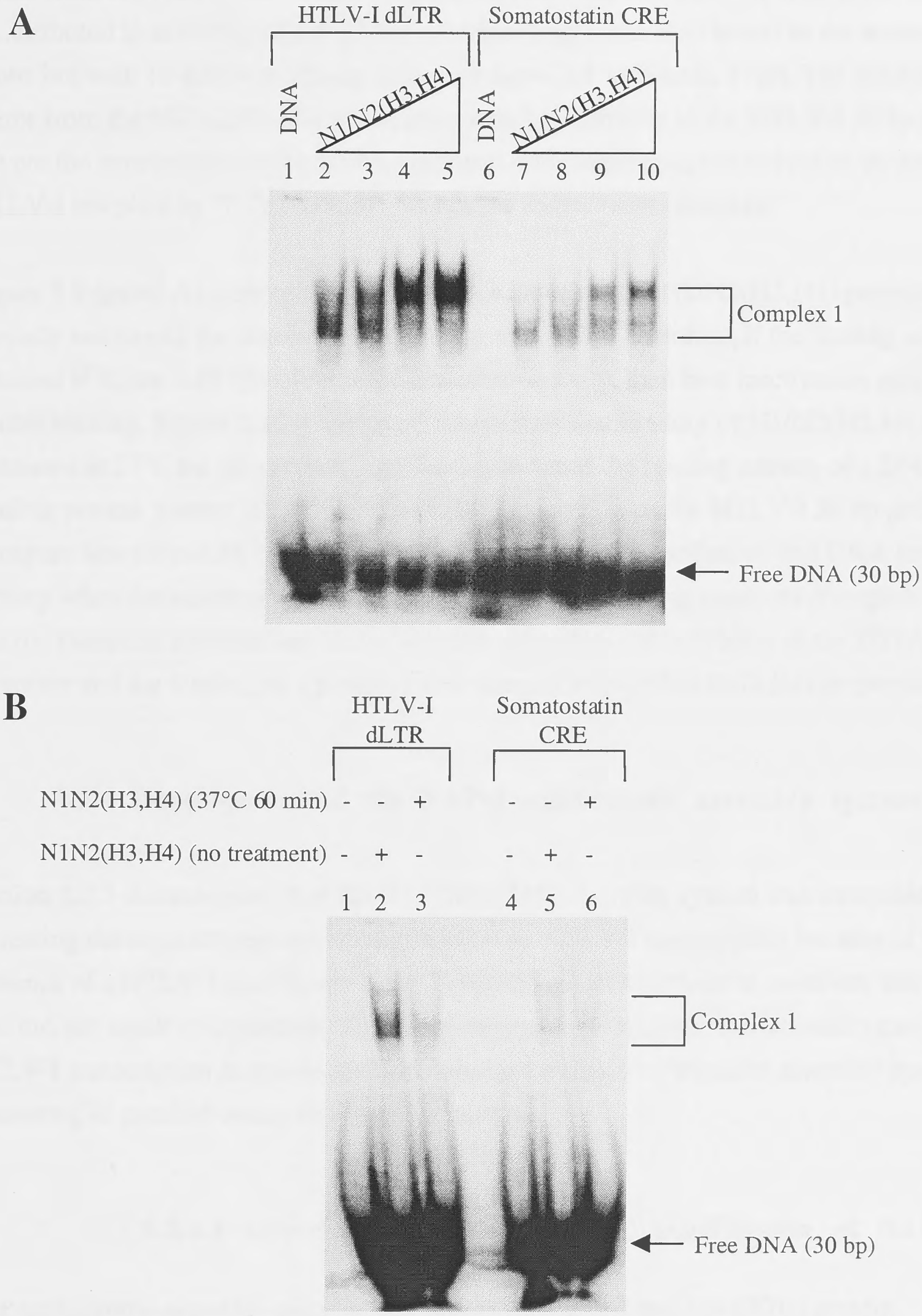


Figure 5.10: A component of the N1/N2(H3,H4) complex preparation binds with high affinity to the HTLV-I LTR. A. Increasing concentrations of the N1/N2(H3,H4) preparation were bound to the 30 bp HTLV-I dLTR probe (lanes 1-5) and the 30 bp somatostatin CRE probe (lanes 6-10). Lanes 1 and 6: Free DNA; lanes 2-5 and 7-10: 5, 10, 15 and 20 µl of N1/N2(H3,H4). Free DNA is indicated by an arrow and the mobility shift is indicated as complex 1. **B.** Heat treatment of N1/N2(H3,H4) complex results in a loss of complex 1 binding activity. N1/N2(H3,H4) was either untreated (lanes 2 and 5) or incubated at 37°C for 60 min (lanes 3 and 6) and bound to either the 30 bp HTLV-I probe (lanes 1-3) or the somatostatin probe (lanes 4-6). Lanes 1 and 4: Free DNA. Free DNA is indicated by an arrow and the mobility shift is indicated as complex 1.

factor from N1/N2(H3,H4) to the 30 bp probe. Note that this mobility shift is too small to be attributed to assembly of the H3/H4 tetramer. This factor also bound to the somatostatin probe but with 10-fold less affinity (compare lanes 2-5 with lanes 7-10). The binding of a factor from the N1/N2(H3,H4) preparation with high affinity to the HTLV-I 30 bp probe, but not the somatostatin 30 bp probe, correlates with transcriptional activation of the HTLV-I template by N1/N2(H3,H4) but not the somatostatin template.

Figure 5.9 (panel A) demonstrated that heat treatment of the N1/N2(H3,H4) preparation partially inactivates the contaminating activation activity. Therefore, if the binding activity detected in figure 5.10 (panel A) is the activation activity, then heat inactivation may also inhibit binding. Figure 5.10 (panel B) demonstrates that binding of N1/N2(H3,H4), pre-incubated at 37˚C for 60 minutes, significantly reduced the binding activity of a DNA binding protein present in the N1/N2(H3,H4) preparation on the HTLV-I 30 bp probe (compare lanes 2 and 3). Furthermore, heat activation had no effect on this DNA binding activity when the somatostatin 30 bp probe was used in binding reactions (compare lanes 5 and 6). Therefore a correlation exists between transcriptional activation of the HTLV-I promoter and the binding of a protein factor present in the N1/N2(H3,H4) preparation.

5.2.4 Development of the NAP-1 nucleosome assembly system

Section 5.2.3 demonstrated that the N1/N2(H3,H4) assembly system was incapable of recreating the expected repressive environment on HTLV-I transcription because of the presence of a HTLV-I specific activator. In addition, heat treatment to inactivate this activity also did not result in repression. Therefore, to determine if chromatin assembly can repress HTLV-I transcription as it does for somatostatin template, a chromatin assembly system consisting of purified components was developed.

5.2.4.1 Sub-cloning, expression and purification of NAP-1

The nucleosome assembly protein NAP-1 was sub-cloned into the 6XHis protein expression vector to create pET-NAP as described in the methods (section 2.11.1.7a) and a map of pET-NAP is shown in figure 5.11 (panel A). Recombinant 6XHis-NAP was expressed in *E.coli* and purified by nickel affinity chromatography under denaturing conditions (section 2.11.1.7). Figure 5.11 (panel B) shows the elution profile of NAP-1 from the nickel column.

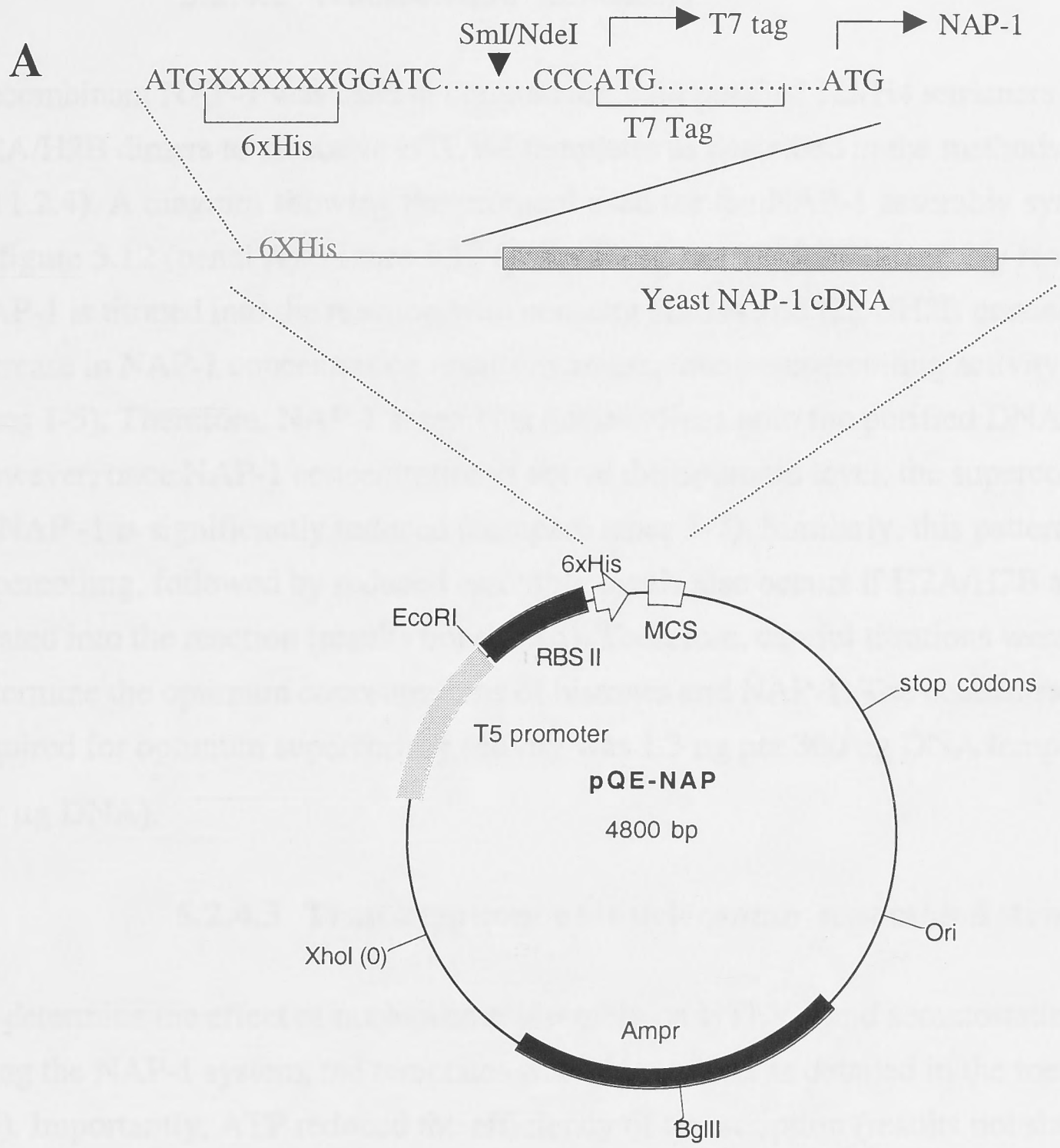


Figure 5.11: Sub-cloning and purification of recombinant yeast NAP-1.
A. The cDNA for yeast NAP-1 (Fujii-Nakata *et al.*, 1992) (including the first 11 amino acids of the T7 gene 10 product) was sub-cloned in frame with the 6 histidine (6XHis) tag from the pQE vector to create pQE-NAP. The *Sma*I/*Nde*I ligation site, the start of the T7 promoter and the NAP-1 protein are indicated. **B.** Purification of NAP-1. NAP-1 was expressed in *E.coli* from pQE-NAP and the cell lysates were denatured in 8M urea and purified by nickel column chromatography. The 100 mM imidazole elution profile from the nickel column is shown (lanes 1-9). Molecular weight markers (kDa) are shown to the left of the figure.

5.2.4.2 Nucleosome assembly

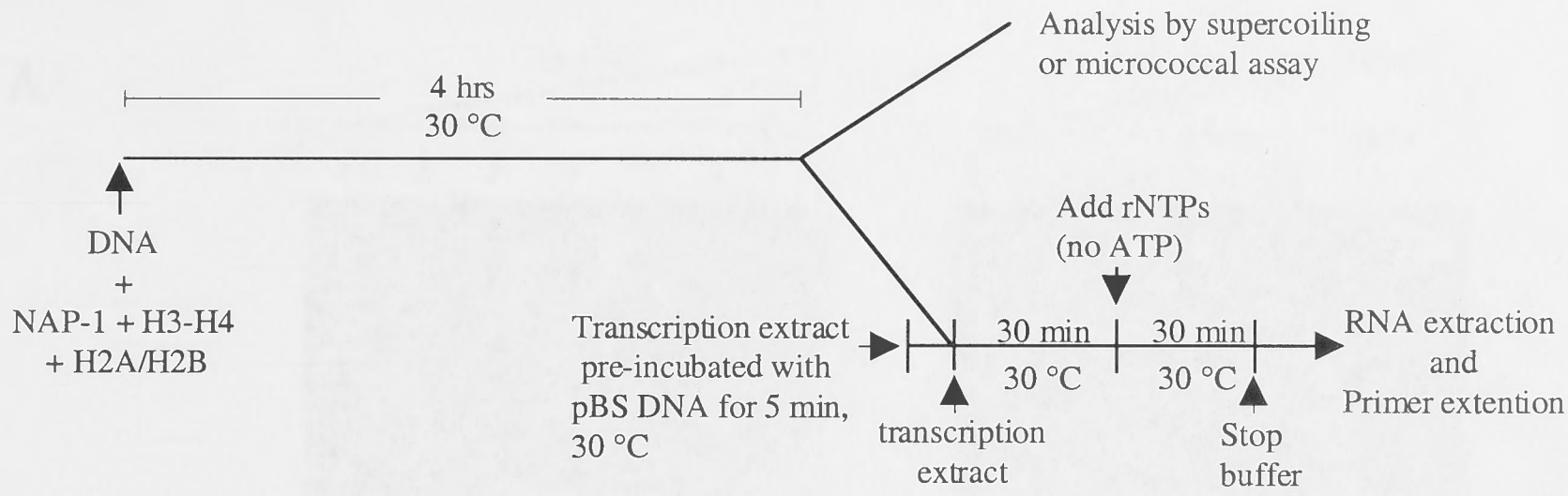
Recombinant NAP-1 was used in conjunction with purified H3/H4 tetramers and H2A/H2B dimers to assemble HTLV-I templates as described in the methods (section 2.11.2.4). A diagram showing the protocol used for the NAP-1 assembly system is shown in figure 5.12 (panel A). Figure 5.12 (panel B) shows a NAP-1 assembly reaction where NAP-1 is titrated into the reaction with constant H3/H4 and H2A/H2B concentrations. An increase in NAP-1 concentration results in an increase in supercoiling activity (compare lanes 1-5). Therefore, NAP-1 assembles nucleosomes onto the purified DNA templates. However, once NAP-1 concentration is above the optimum level, the supercoiling activity of NAP -1 is significantly reduced (compare lanes 5-7). Similarly, this pattern of optimum supercoiling, followed by reduced assembly levels also occurs if H2A/H2B and H3/H4 are titrated into the reaction (results not shown). Therefore, careful titrations were performed to determine the optimum concentrations of histones and NAP-1. The concentration of NAP-1 required for optimum supercoiling activity was 1.3 ng per 300 ng DNA template (4.3 ng per μg DNA).

5.2.4.3 Transcription of nucleosome assembled templates

To determine the effect of nucleosome assembly on HTLV-I and somatostatin transcription using the NAP-1 system, the templates were transcribed as detailed in the methods (section 2.5). Importantly, ATP reduced the efficiency of transcription (results not shown) and was therefore not added with the other rNTPs. The ATP for transcription comes from the ATP added for the assembly reaction (section 2.5.5). In addition, transcription could only be detected when non-specific competitor DNA was incubated with the Jurkat transcription extract 5 min prior to the transcription reaction (figure 5.12). A supercoiling gel showing the assembly of HTLV-I and somatostatin templates by titration of H3/H4 (in the presence of a constant amount of H2A/H2B) into the assembly reaction is shown in figure 5.13. Analogous to the N1/N2(H3,H4) assembly system, nucleosome density of the assembled templates increased according to the ratio of H3/H4 to DNA (compare lanes 1-7 and 8-14).

To determine the effect of increasing nucleosome density on transcription from the HTLV-I and somatostatin templates, the same templates shown in panel A were transcribed *in vitro*. Figure 5.13 (panel B) shows that assembly using the purified NAP-1 system represses transcription from both the somatostatin (lanes 8-14) and HTLV-I (lanes 1-7) templates. Therefore, using purified assembly conditions, HTLV-I transcription is repressed by

A



B

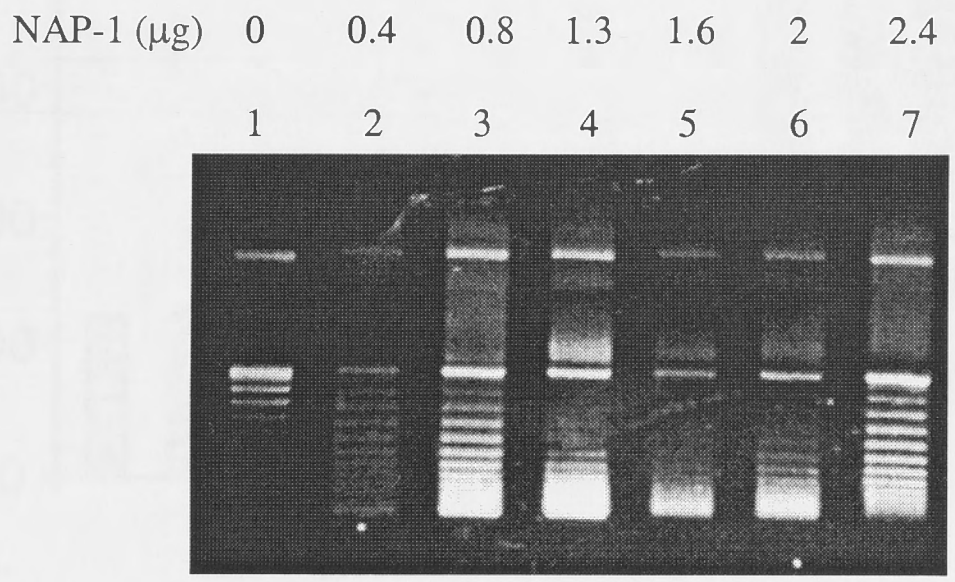


Figure 5.12: Nucleosome assembly using the NAP-1 assembly system.
A. Schematic diagram showing the protocol followed for assembly of promoter templates with the purified NAP-1 assembly system. **B**. Supercoiling analysis of HTLV-I template DNA assembled using increasing concentrations of NAP-1. Lanes 1-7 each contain 300 ng HTLV-I template DNA, 2 U topoisomerase I, 0.5 μl of purified H3-H4 tetramer and 1.5 μl H2A/H2B (both purified from long chicken chromatin). The concentrations of NAP-1 are shown above each lane.

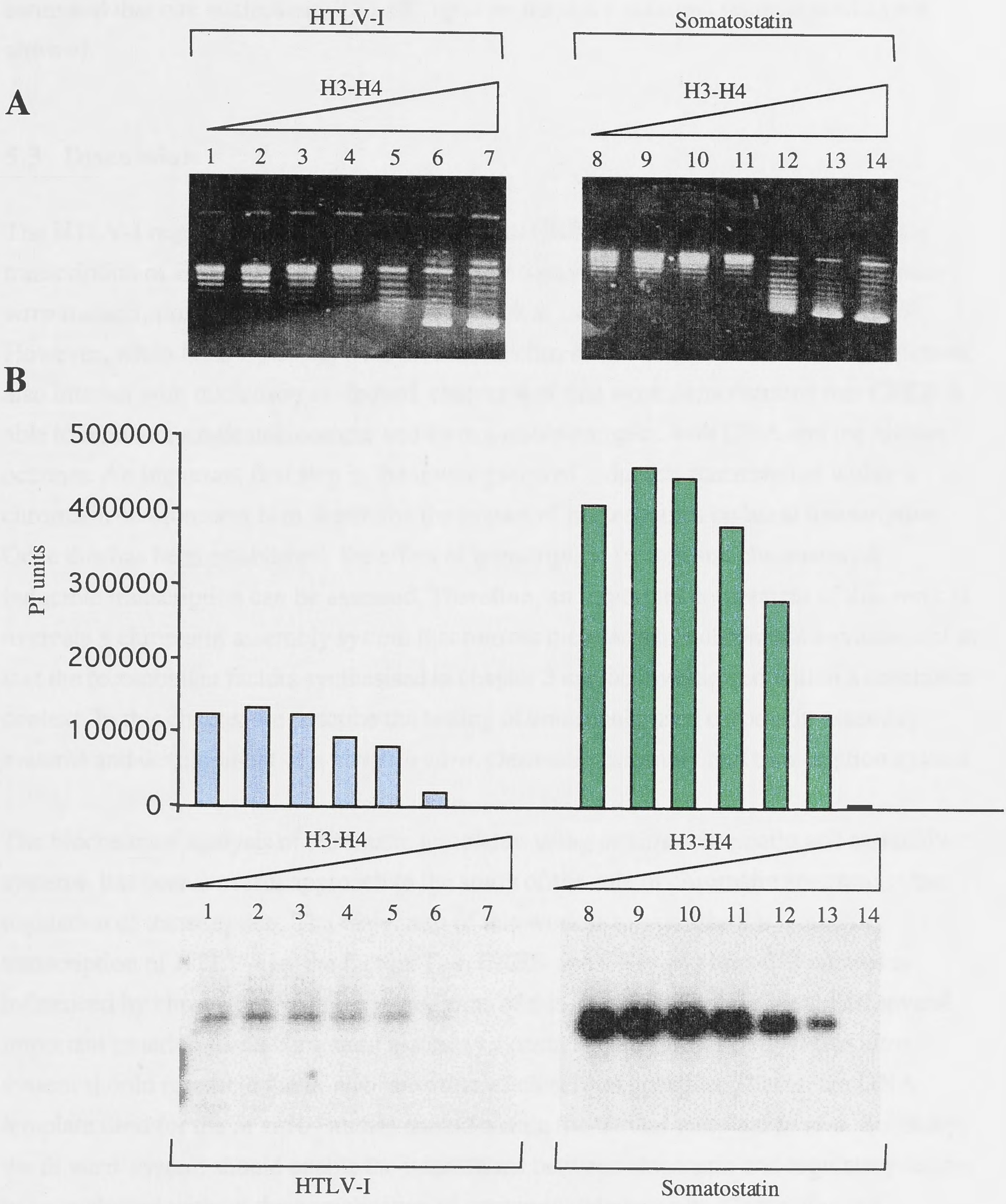


Figure 5.13: nucleosome assembly using the NAP-1 system represses the basal transcription of HTLV-I. 300 ng of HTLV-I (left panel) or somatostatin (right panel) template DNA was assembled using the NAP-1 system with increasing concentrations of H3-H4 before analysis of the templates by *in vitro* transcription. **A.** Supercoiling analysis of the assembled templates. Lanes 1-7 and 8-14 contained 1.3 µg NAP-1, 1.5 µl H2A/H2B and 0, 0.05, 0.2, 0.1, 0.5, 1 and 2 µl of H3-H4 respectively. **B.** *In vitro* transcription from the same templates shown in panel A. Lanes as for panel A. Products of the *in vitro* transcription were analysed by primer extension, resolved on a sequencing gel and exposed to a phosphoimage screen. The bands were quantitated using image gauge software and are shown above the figure. Units are expressed as phosphoimage (PI) units.

nucleosome assembly. Furthermore, like the N1/N2(H3,H4) assembly system, it is estimated that one nucleosome/160-180 bp is required for maximal repression (data not shown).

5.3 Discussion

The HTLV-I regulatory proteins Tax, CREB and CBP have been shown to regulate the transcription of HTLV-I in transient transfection assays or as naked DNA templates in *in vitro* transcription assays (for example see Kwok *et al.*, 1996; Lenzmeier *et al.*, 1988). However, when HTLV-I exists within a natural chromatin environment, these factors must also interact with nucleosomes. Indeed, chapter 4 of this work demonstrated that CREB is able to bind to a single nucleosome and form a stable complex with DNA and the histone octamer. An important first step in the investigation of inducible transcription within a chromatin environment is to determine the impact of nucleosomes on basal transcription. Once this has been established, the effect of transcription factors and chromatin on inducible transcription can be assessed. Therefore, an important component of this work is to create a chromatin assembly system that mirrors the repressive chromatin environment so that the recombinant factors synthesised in chapter 3 can be investigated within a chromatin context. In this chapter we describe the testing of commonly used chromatin assembly systems and development of a novel *in vitro* chromatin assembly and transcription system.

The biochemical analysis of chromatin templates, using *in vitro* chromatin and assembly systems, has been a useful approach to the study of the role of chromatin structure in the regulation of transcription. The major aim of this work is to examine the inducible transcription of HTLV-I by the factors Tax, CREB and CBP, and how this control is influenced by chromatin. With the major aims of this work in mind, we identified several important criterion that a chromatin assembly system should fulfil. Firstly the *in vitro* system should resemble the *in vivo* situation as closely as possible. That is, the DNA template used for the *in vitro* studies should mimic the natural template *in vivo*. Secondly, the *in vitro* system should enable the interactions between chromatin and regulatory factors to be evaluated without the complication of contaminating proteins. Contaminating transcription factors may influence the transcription process masking the function of regulatory proteins of interest on transcription.

An example of a chromatin assembly system is the salt transfer method of nucleosome assembly used in chapter 4 of this work to assemble small DNA fragments into a single nucleosome. Variations on this system can assemble nucleosomes onto larger pieces of DNA (Ng *et al.*, 1997). This system uses purified histones without the need for assembly factors or ATP and therefore, does not contain contaminating proteins that may influence transcription. A major limitation of the salt transfer method, however, is that because of the high salt concentration required to transfer histones, transcription factors cannot be added to the DNA at the time of histone assembly but can only be added after assembly as taken place. Competition between transcription factors and histones for key regulatory DNA sequences is thought to play a important role in the regulation of gene expression (Brown, 1984; Tremethick *et al.*, 1990). Therefore, this limitation restricts the utility of the salt transfer method to fully understand nucleosome-factor interactions in transcriptional control.

Cell free protein extracts overcome the limitation of the salt transfer method as transcription factors can be added both prior to and after nucleosome assembly. The S-190 and S-150 *Drosophila* and *Xenopus* extracts, respectively, have been used extensively as chromatin assembly systems to investigate inducible transcription by factors such as GAL4 derivatives (Pazin *et al.*, 1994), LEF-1 (Sheridan *et al.*, 1995) NF-κB proteins (Pazin *et al.*, 1996), oestrogen receptor (Kraus and Kadonaga, 1998) and CREB (Mayall *et al.*, 1997). However, in this study we have demonstrated that these crude protein extracts contain factors that can support transcription, even in the absence of a transcription extract. In addition, these factors mediate the high level activation of transcription in the presence of transcription extract (section 5.2). Other investigators have also noted the presence of these factors and moreover, suggest that these factors are important for transcription of the chromatin template (Pazin *et al.*, 1998 and 1994; Ito *et al.*, 1997; Kraus and Kadonaga, 1998). For example, Pazin *et al.* (1998) has shown that a GAL4 derivative, GAL4-VP16, binds to chromatin with comparable affinity to its binding to naked DNA using the *Drosophila* S-190 assembly system. However, using salt transfer, Taylor *et al.* (1991) showed a 100 fold difference in binding affinity between chromatin and naked DNA. These contradictory results may relate to the ability of the S-190 system to physiologically space nucleosomes or the presence of factors in the S-190 extract that can mediate the binding of GAL4-VP16 to nucleosomes. Indeed, activator and ATP-dependent chromatin remodelling or nucleosome spacing complexes such as NURF (Tsukiyama and Wu, 1995; Tsukiyama *et al.*, 1995), ACF (Ito *et al.*, 1997) and CHRAC (Varga-Weisz *et al.*, 1997) have been

identified by fractionation of these extracts. In this chapter, the presence of the S-150 factors inhibited the ability of nucleosomes to repress the basal transcription of HTLV-I and somatostatin templates. Therefore the S-150 assembly system could not be used to determine the effect of recombinant factors on the de-repression of chromatin for HTLV-I transcription in this study.

Derivatives of protein extracts have also been used extensively for chromatin assembly. For example, heat treated supernatant from *Xenopus* eggs can be used as a source of nucleosome assembly factors that will assemble nucleosomes onto purified DNA when supplemented with purified histones (Laskey *et al.*, 1978; Workman *et al.*, 1988). In this study the N1/N2(H3,H4) complex, partly purified from the S-150 extract, was used to assemble nucleosomes on HTLV-I and somatostatin templates. The N1/N2(H3,H4) complex in combination with purified H2A/H2B dimers has been used to successfully assemble chromatin on many DNA templates including the major late promoter from adenovirus and the dihydrofolate reductase (DHFR) promoter (Tremethick and Frommer, 1992). Indeed, this system was able to repress the transcription from chromatin assembled somatostatin templates (figure 5.7). However, a contaminant in the N1/N2(H3,H4) complex specifically activated the transcription of the HTLV-I LTR. Therefore, for the same reasons discussed for the S-150 extract, the N1/N2(H3,H4) system was not appropriate for use in the *in vitro* analysis of HTLV-I transcription on chromatin templates in this study.

The identification of an activating activity which appears to be specific for the HTLV-I promoter in the S-150 extract is a potentially interesting finding. This chapter has shown that this activation activity can be strongly correlated to a DNA binding activity in the N1/N2(H3,H4) complex. This correlation suggests that the activating activity may be a DNA binding transcription factor. The sequence of the TRE elements suggests that the transcription factor maybe a member of the ATF/CREB or AP-1 families of bZIP transcription factors. However, this factor cannot bind to the somatostatin CRE with a very high affinity and this type of preference for the HTLV-I binding site has not been demonstrated for any bZIP proteins. Indeed, the protein thought to be responsible for Tax mediated HTLV-I transcription, CREB, binds the somatostatin CRE with a greater affinity than the HTLV-I TRE (section 3.2.1.2). The identity of this factor in the *Xenopus* S-150 extract may enable the analogous protein to be identified in the human and may have potential importance in the control of HTLV-I transcription. For example, this factor may

act in combination with Tax, CREB, CBP and other factors to cause high level expression of the virus.

The inability of the S-150 extract and the more purified N1/N2(H3,H4) system to create a repressive chromatin environment for *in vitro* transcription necessitated the development of an assembly system that utilised purified components. Several systems have previously been developed that also reconstitute chromatin from purified components. These systems use negatively charged molecules including polyglutamic acid (Stein *et al.*, 1979) and RNA (Nelson *et al.*, 1981). These negatively charged molecules prevent the aggregation pathways encountered on directly mixing the components at physiological salt concentrations. A modified form of the salt transfer method, which enables assembly of factors prebound to DNA, has also been used as a purified assembly system (Chang and Luse 1997). In addition, chromatin assembly factors such as nucleoplasmin (Rhodes *et al.*, 1989) have been used to assemble purified histones onto DNA templates. These purified systems avoid the possible introduction of chromatin remodelling factors from extracts.

In this study, the chromatin assembly factor yeast NAP-1, was N-terminally tagged with 6XHis and purified by nickel chromatography. Fujii-Nakata *et al.* (1992) have also purified a recombinant yeast NAP-1, tagged with the T7 gene product. However, purification of this protein results in the co-purification of nuclease activity (Dr J. Kadonaga, personal communication). The yeast NAP-1 was chosen for use as a chromatin assembly factor as it exhibits a greater propensity to deposit histones onto purified DNA templates than *Drosophila* NAP-1 (Ito *et al.*, 1996). In this study, purified core histones from chicken long chromatin (sections 2.11.1.5 and 2.11.1.6) were used to assemble nucleosomes. However NAP-1 is also able to assemble recombinant histones onto purified DNA templates (results not shown). The concentration of NAP-1 required for optimal supercoiling activity in this study was 4.3 ng/µg DNA template. Therefore, the 6XHis yeast NAP-1 in this study assembles nucleosomes at comparable concentration to T7 tagged yeast NAP-1 (5 ng/µg DNA template) (Fujii-Nakata *et al.*, 1992) and *Drosophila* NAP-1 (8 ng/µg DNA template) (Ito *et al.*, 1996).

The purified NAP-1 assembly system developed in this study was used to assemble nucleosomes that repressed transcription from HTLV-I and somatostatin templates. Therefore, the use of this system has shown that HTLV-I transcription can be repressed by nucleosome assembly. In addition, this system can be used to investigate the effect of

transcription factors on this repressive chromatin environment. Therefore, the NAP-1 system fulfils a major requirement for an *in vitro* chromatin assembly and transcription system in this study. One possible drawback of this system, as well as the purified systems discussed above, is that these systems do not physiologically space nucleosomes. However, since ATP dependent chromatin remodelling complexes are spacing factors, the added complication exists of whether the introduced transcription factors of interest are alone sufficient for transcriptional activation. The next step to further develop the NAP-1 system would be to introduce purified spacing activity such as that isolated by Guschin *et al.* (2000). The differences in nucleosome deposition between purified systems and methods that utilise crude extracts is thought to effect the properties of the assembled templates. For example, significant differences have been shown in the extent of transcriptional repression by histone H1 with chromatin that was assembled with a purified system, relative to chromatin that was assembled using the S-190 extract (Pazin *et al.*, 1994; Kamakaka *et al.*, 1993; Laybourn and Kadonaga, 1991). Differences in the spacing of the nucleosomes by purified versus protein extract systems could be investigated using the NAP-1 assembly system by the addition of a purified ATP dependant spacing activity to the purified assembly systems (Guschin *et al.*, 2000; Tremethick and Frommer, 1992).

In vitro transcription can also be accomplished with purified components as well as protein extracts. For example, *in vitro* transcription experiments with nucleosomal templates from purified assembly and transcription systems reveal that the core RNA polymerase II cannot transverse through nucleosomes (Chang and Luse, 1997). Importantly this result suggests that *in vivo*, the association between RNA pol II and accessory proteins may be important for template traversal. Indeed, fractionation of transcription extracts has identified factors that are required for the ability to transcribe nucleosomal templates. For example Orphanides *et al.*, (1998) has shown that transcriptional elongation on chromatin templates requires the presence of FACT (facilitates chromatin transcription). Wada *et al.*, 1998 have identified a component of the HeLa nuclear extract that causes pausing of RNA Pol II in conjunction with a transcription inhibitor, called DSIF. Furthermore, a factor also identified in HeLa nuclear extract, termed TAF-1, has been shown to activate transcription from the adenovirus promoter by chromatin remodelling (Okuwaki and Nagata, 1998). Interestingly, TAF-1 was shown to be structurally related to NAP-1 and has NAP-1 activity (Kawase *et al.*, 1996). Therefore TAF-I may be important for chromatin remodelling activities that activate transcription and be involved in nucleosome assembly.

For the majority of *in vitro* transcription studies on naked and assembled DNA presented in this thesis, nuclear extract derived from Jurkat T-cells was used to provide the general transcription machinery. The transcription extract was generated using the Dignam protocol (Dignam *et al.*, 1983) but with the modification of precipitating histones and non-histone proteins from the extract with ammonium sulphate 3.6 g/µl (section 2.5.4). This method of extract preparation has been used extensively by many investigators and contains RNA polymerase II and additional components of the general transcription machinery required for transcription. However, *in vitro* transcription is not as efficient as transcription *in vivo* and therefore, some important factors may be missing. It has been reported that *in vitro*, a unique RNA polymerase, with characteristics of RNA polymerases II and III is capable of transcription of the HTLV-I promoter (Piras *et al.*, 1994). This was based on the findings that HTLV-I transcription is resistant to the RNA pol II inhibitor α-amanitin (Duvall *et al.*, 1995; *Piras et al* 1994). This chapter has shown that RNA transcription is sensitive to α-amanitin and is therefore a result of RNA poll II transcription. A major difference in the methodology between this work and Piras *et al.* (1994) is that the later use whole cell extract whereas the former (this study) use nuclear extract. The sensitivity of HTLV-I transcription has also been noted by other investigators (Lenzmeier and Nyborg 1997; Matthews *et al.*, 1993). Perhaps whole cell extract contains factors not present in the nuclear extract that cause insensitivity to α-amanitin.

In this chapter, we have developed an *in vitro* chromatin assembly system using purified components and used this system in combination with the Jurkat transcription extract to create an *in vitro* chromatin and transcription system. Using this system, we have shown that nucleosome assembly represses basal transcription on the HTLV-I promoter. The overall aim of this work is to examine the role of chromatin in the inducible expression of HTLV-I. The development of the NAP-1 assembly system that is free of contaminating activating activities, in addition to the use of full length proteins and natural promoter sequences represents a new approach to investigate the regulation of inducible HTLV-I transcription and will be the focus of chapter 6 of this thesis.

CHAPTER 6:

The effect of factors and chromatin on the inducible transcription of HTLV-I in an *in vitro* chromatin assembly and transcription system.

6.1 Introduction

The mechanisms by which transcription factors can activate transcription can be divided into three categories.

1) True activation: Activators can facilitate the inherent transcription reaction by forming direct contacts between activators and components of the general transcription machinery. Such contacts have been proposed to regulate pre-initiation complex formation and DNA melting at the promoter (Ptashne and Gann, 1990; Kingston and Green, 1994). In addition, activators may also play a direct role in the regulation of elongation. For example, a variety of activators are able to stimulate elongation through pausing and termination sites on stably transfected reporter constructs (Yankulov *et al.*, 1994). Furthermore, enhancers can increase read-through of promoter-proximal pausing (Krumm *et al.*, 1995).

2) Derepression from chromatin templates: Studies on chromatin templates show that activators can relieve nucleosomal inhibition of transcription, possibly by weakening histone-DNA contacts or through contacts with complexes like SWI/SNF that modify chromatin structure (Travers, 1999). With repressed chromatin templates, transcriptional activation by a sequence specific factor is a combination of both derepression and true activation.

3) Non-activators: The existence of co-factors that can increase affinity and stability of transcriptional activators for their DNA binding site provides an alternative strategy by which DNA binding proteins can effectively compete with histones for naked DNA. Examples of this form of factor are HMG-I(Y) on the INF-β enhanceosome (Falvo *et al.*, 1995), LEF-1 at the mouse T-cell receptor α-gene enhancer (Giese and Grosschedl, 1993; Carlsson *et al.*, 1993) and YY1 on the *c-fos* gene promoter (Natesan and Gilman, 1993). These mechanisms are not mutually exclusive and high level transcriptional activation is likely to result from a combination of these mechanisms.

This study uses the HTLV-I promoter and the key factors that interact with it as a model for inducible transcription within a chromatin environment. HTLV-I transactivation is thought to require the assembly of Tax and CREB on three 21-bp repeat elements located in the U3 region of the HTLV-I promoter (figure 1.8). The HTLV-I encoded protein Tax has been shown to activate HTLV-I transcription in tissue culture reporter assays and in *in vitro* transcription assays on naked DNA templates (Lenzmeier *et al.*, 1998; Sodroski *et al.*, 1984; Matthews *et al.*, 1992; Lenzmeier and Nyborg, 1997). However, recent work by Kashanchi *et al.* (1998) showed that transcription from the HTLV-I promoter was not activated by the addition of Tax in *in vitro* transcription analysis. Work presented in this study (section 3.2.2.2 and 3.2.3) and many published studies, have demonstrated that Tax increases the DNA binding activity of CREB. The association between Tax and CREB is thought to produce a ternary complex on the HTLV-I LTR. However, the mechanism by which the complex results in the activation of transcription remains unclear. The recruitment of Tax to the HTLV-I promoter by CREB may position Tax to interact with basal transcription factors. In support of this model, Tax has been shown to interact with the basal transcription factors TBP and $TAF_{II}28$ (Caron *et al.*, 1993; 1997), TFIIA (Clemens *et al.*, 1996) and TFIID (Duvall *et al.*, 1995).

The transcriptional coactivator, CBP was first identified as a component of the CREB activation pathway (Kwok *et al.*, 1994; Chrivia *et al.*, 1993). At the conception of this study in 1996, we set out to determine whether CBP was also involved in HTLV-I transcription. Indeed, recent studies have shown that CBP can interact with Tax *in vitro*, which strengthened our hypothesis that Tax, CREB and CBP form a quaternary complex with the HTLV-I LTR (Kwok *et al.*, 1996; Giebler *et al.*, 1997). In the presence of Tax, CBP binding and transactivation of the LTR do not require CREB to be phosphorylated (Parker *et al.*, 1996; Kwok *et al.*, 1996), presumably CBP is recruited through Tax and not through the activation domains of CREB.

The presence of CBP in the HTLV-I transcription complex provides additional possible mechanisms for gene activation. CBP has been reported to interact with the basal transcriptional machinery (Swope *et al.*, 1996; Bisotto *et al*, 1996; Dallas *et al.*, 1997). Furthermore, the N-terminal half of CBP, which contains the TBP binding site (figure 1.7) has been shown to be important for CBP mediated transcription (Swope *et al.*, 1996). It has also been reported that CBP can interact with the RNA polymerase holoenzyme and the histone acetyltransferases p/CAF, in addition to containing an intrinsic HAT domain (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996). Martinez-Balbas *et al.* (1998) has

demonstrated that the acetylation activity of CBP is required for the activation activity of CBP but only on certain promoters. With regard to the function of CBP in HTLV-I transcription, it is unknown which, if any of these functions of CBP contribute to gene activation.

In vitro, the assembly of promoter elements into chromatin is critical to the function of some genes. For example, inducible transcription by the ER is observed on chromatin templates but not on non-chromatin templates (Kraus and Kadonaga, 1998). Furthermore, long range activation by GAL4-VP16 was observed on chromatin assembled templates and not naked DNA (Laybourn and Kadonaga, 1992; Schild *et al.*, 1993). Clearly chromatin plays an important role in the regulation of gene transcription. Therefore, studies of naked DNA templates in *in vitro* transcription experiments may not represent the true mechanism of transcriptional regulation seen on chromatin *in vivo*.

Published studies concerning the interaction of Tax, CREB and CBP with the HTLV-I LTR has been on naked DNA templates only. However, the natural environment for the HTLV-I promoter is within the chromosome of infected cells. With regard to chromatin templates, we have demonstrated that CREB is a high affinity nucleosome binding protein which can bind to the nucleosome without disturbing nucleosome structure (section 4.2.2). Several published studies have investigated the role of some of the HTLV-I interacting factors on chromatin templates. For example Mayall *et al.* (1997) have demonstrated that phosphorylated CREB can activate the chromatin assembled TCRα enhancer *in vitro*. Kraus and Kadonaga, (1998) have shown that p/300 can activate inducible expression by the oestrogen receptor on a chromatin assembled template *in vitro*. Both these studies employed the Drosophila S-190 chromatin assembly system which contains many factors that may influence transcription (chapter 5). In this chapter we use the purified NAP-1 assembly system to investigate the role of chromatin on the inducible transcription of HTLV-I by the factors Tax, CREB and CBP in the absence of contaminating factors like remodelling complexes present in the S-190 *Drosophila* extract. Throughout the course of this study we also observed specific interaction between the HMG protein HMG-I(Y) and the HTLV-I LTR. Although this work is preliminary, a possible function for HMG-I(Y) in the control of HTLV-I transcription is also investigated.

6.2 Results

6.2.1 The effect of Tax, CREB and CBP on the transcription of maximally assembled chromatin templates

To investigate the effect of factors on transcription from nucleosome assembled templates, 300 ng of HTLV-I and somatostatin template DNA were assembled into nucleosomes using NAP-1 and purified core histones (section 5.2.4). A diagram of the protocol used for the assembly and *in vitro* transcription reactions is shown in figure 6.1 (panel A). Chapter 5 showed that the nucleosome density on the purified DNA templates was dependant on the ratios of NAP-1, H3,H4 and H2A/2B to each other and to the template DNA (section 5.2.4). Moreover, the maximum level of nucleosome assembly attainable using the NAP-1 assembly system completely repressed transcription. Therefore, to determine if the factors Tax, CREB and CBP alone, or in combination, are sufficient to activate transcription from this highly repressed state, the concentrations of each assembly component was empirically determined so that maximum assembly occurs according to the supercoiling assay (results not shown). Figure 6.1 (panel B) shows a supercoiling assay of the same nucleosome assembled template used for *in vitro* transcription of HTLV-I templates in figure 6.2 (panel A) indicating that the template DNA is highly supercoiled.

Using the NAP-1 assembly system, recombinant factors can be added to the template DNA either at the time of nucleosome assembly or after. Published studies investigating the effect of CREB (on the TCRα promoter) and CBP (on an artificial 4 x vitellogeninA2/Adenovirus E4 core promoter) by Mayall *et al.* (1887) and Kraus and Kadonaga, (1998) respectively, have shown that CREB or p/300/CBP added to the template DNA at the onset of the assembly reaction, are able to activate transcription on chromatin assembled templates. Therefore, we investigated whether CBP can enhance HTLV-I expression. In this study, factors were also added to the template DNA with the components of the assembly system. The concentrations of each of the factors; Tax, CREB and CBP, were empirically determined to give maximum transcription from naked DNA templates (results not shown). Preliminary supercoiling assays indicated that addition of CREB and Tax at the time of nucleosome assembly inhibited the activity of topoisomerase 1 (results not shown). To overcome this problem, DNA was preincubated with topoisomerase I at 30°C for one hour prior to its addition to the nucleosome assembly reaction (figure 6.1, panel A). Figure 6.1 (panel B) shows that addition of Tax and CBP at the start of the assembly reaction had no

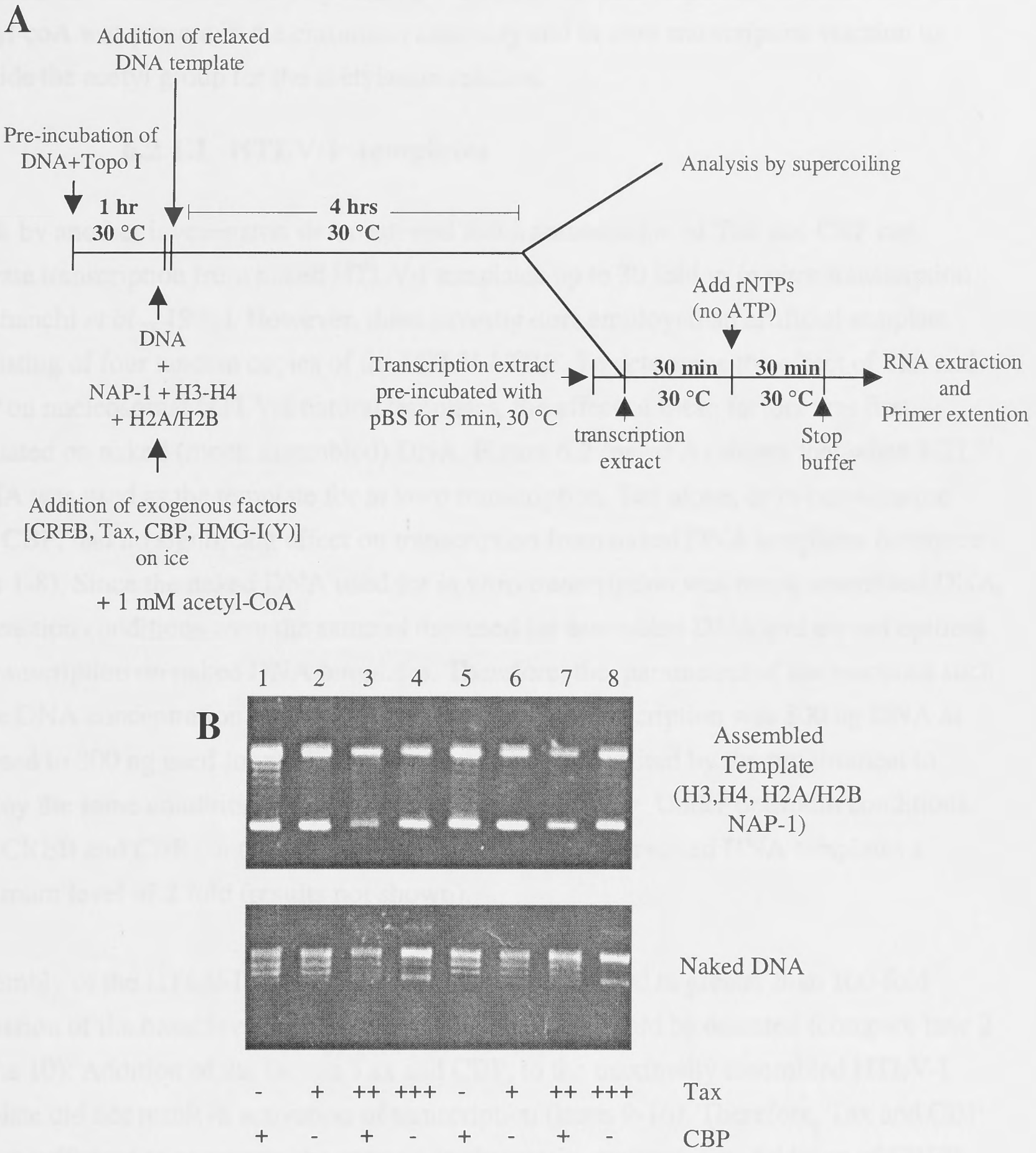


Figure 6.1: Assembly of templates for inducible transcription. A. Schematic diagram representing the protocol employed for the assembly of HTLV-I and somatostatin templates. Recombinant transcription factors including Tax, CREB and CBP were added to the DNA with the components of the assembly reaction. **B**. Supercoiling analysis of HTLV-I templates used in the *in vitro* transcription reaction shown in figure 6.2**.** Each lane contains 300 ng of HTLV-I template. Assembled lanes (top panel) contain 1.3 ng NAP-1, 1.5 µl H2A/H2B and 2 µl of H3-H4. Addition of recombinant proteins, Tax [either 0 (-), 1 (+), 3 (++) or 5 (+++) µl] and CBP (100 ng) are indicated below the figure.

effect on the level of supercoiling of HTLV-I templates. It is important to note that 1 mM acetyl-coA was present in the chromatin assembly and *in vitro* transcription reaction to provide the acetyl group for the acetylation reaction.

6.2.1.1 HTLV-I templates

Work by another investigator, demonstrated that a combination of Tax and CBP can activate transcription from naked HTLV-I templates up to 30 fold in *in vitro* transcription (Kashanchi *et al* ., 1998). However, these investigators employed an artificial template consisting of four tandem copies of the HTLV-I TRE. To determine the effect of Tax and CBP on nucleosomal HTLV-I natural templates, the effect of these factors was first evaluated on naked (mock assembled) DNA. Figure 6.2 (panel A) shows that when HTLV-I DNA was used as the template for *in vitro* transcription, Tax alone, or in combination with CBP, had no significant effect on transcription from naked DNA templates (compare lanes 1-8). Since the naked DNA used for *in vitro* transcription was mock assembled DNA, the reaction conditions were the same as that used for assembled DNA and are not optimal for transcription on naked DNA templates. Therefore, the parameters of the reactions such as the DNA concentration (optimum level for HTLV-I transcription was 800 ng DNA as opposed to 300 ng used for nucleosome assembly) were limited by the requirement to employ the same conditions used for nucleosome assembly. Under optimum conditions, Tax, CREB and CBP consistently activated expression from naked DNA templates a maximum level of 2 fold (results not shown).

Assembly of the HTLV-I plasmid into nucleosomes resulted in greater than 100-fold repression of the basal level of transcription as no signal could be detected (compare lane 2 to lane 10). Addition of the factors Tax and CBP, to the maximally assembled HTLV-I template did not result in activation of transcription (lanes 9-16). Therefore, Tax and CBP are not sufficient to overcome the repressive chromatin environment. Addition of CREB also did not enhance transcription (data not shown). It is worth pointing out that in chapter 3, using DNA binding and acetylation assays, Tax, CREB and CBP were active.

6.2.1.2 Somatostatin templates

The highly characterised CREB responsive promoter, somatostatin, was used as a control for inducible transcription in this system. Figure 6.2, (panel B) shows that addition of CREB, CREB+CBP or phosphorylated CREB (P-CREB) and P-CREB+CBP had no effect on the transcription of naked DNA (lanes 1-8). However, any activation of

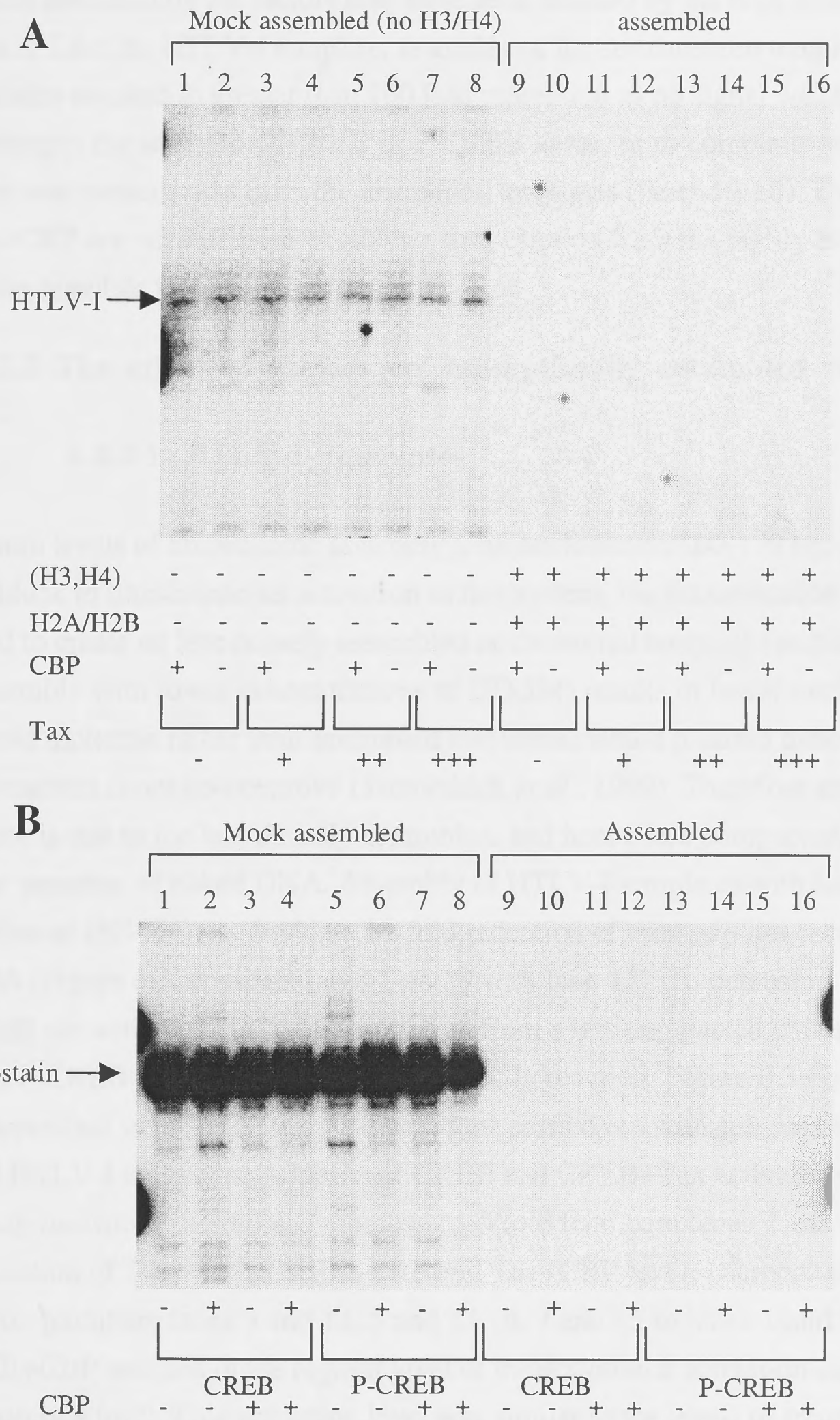


Figure 6.2: The effect of recombinant factors on transcription from naked DNA and maximally assembled nucleosome templates. The maximally assembled HTLV-I (panel A) and somatostatin (panel B) templates shown in figure 6.1 were transcribed *in vitro*. The transcription products were analysed by primer extension and resolved on a sequencing gel. **A.** Each lane contains 300 ng HTLV-I template. Lanes 1-8: Naked DNA; lanes 9-16: 2 µl H3-H4 and 1.5 µl H2A/H2B. Either 0 (-) or 100 (+) ng CBP and either 0 (-), 1 (+), 3 (++) or 5 (+++) µg Tax were added to the assembly reaction as indicated under the figure. **B.** Each lane contains 300 ng of somatostatin template. Lanes 1-8: Naked DNA (mock assembled); lanes 9-16: 1.3 ng NAP-1, 2 µl H3-H4 and 1.5 µl H2A/ H2B. Either 4 µg of CREB or P-CREB, or 100 ng of CBP were added to the assembly reactions as indicated below the figure.

transcription mediated by the factors may have been masked by the high level of transcription. Like the HTLV-I template, assembly of the somatostatin template into chromatin also resulted in greater than 100 fold repression as no signal was detected (lane 9). Surprisingly, the addition of CREB or P-CREB alone, or in combination with CBP, did not activate transcription from the assembled templates (lanes 10-16). Therefore P-CREB and CBP are not sufficient to activate transcription from the highly assembled somatostatin template DNA.

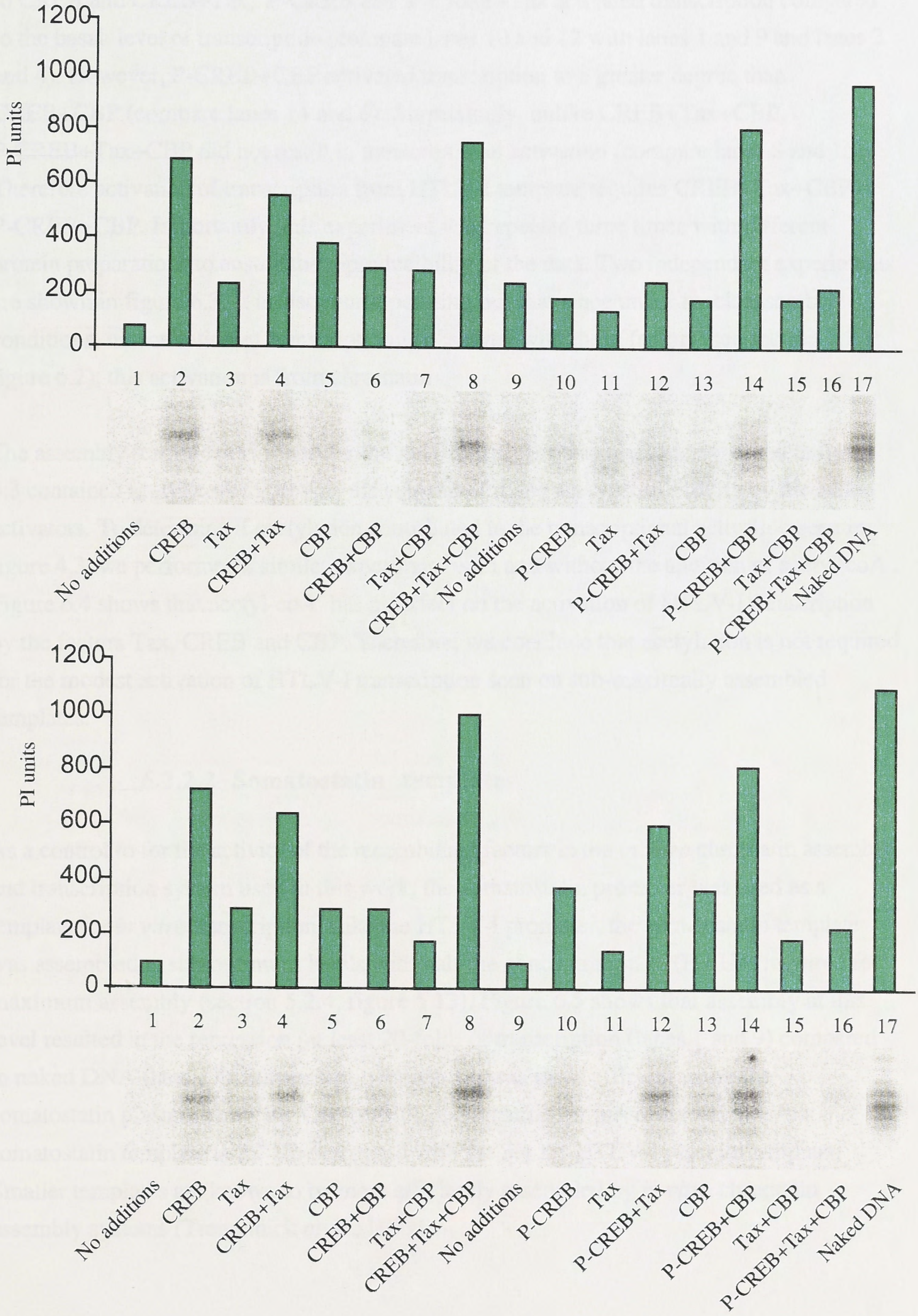
6.2.2 The effect of factors on sup-optimally assembled templates

6.2.2.1 HTLV-I templates

As maximum levels of nucleosome assembly (one nucleosome/160-170 bp) posed a complete block to transcriptional activation in this system, the concentration of (H3,H4) was halved to create an less densely assembled nucleosomal template (section 5.4.1, figure 5.13). Assembly with lower concentrations of (H3,H4) results in fewer nucleosomes on each plasmid molecule rather than assembled and unassembled plasmid molecules since the assembly reaction is not co-operative (Tremethick *et al.*, 1999). Therefore any effect on transcription is due to the less densely assembled, and hence less compacted, chromatin and not the presence of naked DNA. Assembly of HTLV-I templates with half the optimum concentration of (H3-H4) resulted in a 10-fold reduction of transcription compared to naked DNA (Figure 6.3, compare lanes 1 and 9 with lane 17). To determine if exogenously added CREB can activate HTLV-I transcription from a less compacted chromatin structure, CREB and P-CREB was also added to the assembly reaction. Figure 6.3 shows the results of two independent *in vitro* transcription reactions carried out with sub-maximally assembled HTLV-I templates. Addition of CREB and CREB+Tax activated transcription from the sub-maximally assembled templates 4-5 fold (compare lanes 2 and 4 with lanes 1 and 9). Addition of Tax, CBP, CREB+CBP and Tax+CBP had no activation effect on transcription (compare lanes 3 and 11, 5 and 13, 6, 7 and 15 to lanes 1 and 9). Addition of Tax+CREB+CBP resulted in the highest level of transcriptional activation (lane 8) (approximately 8 fold). This activation level was similar to the levels of transcription from naked DNA. (compare lanes 8 and 17). Therefore, this data suggests that Tax+CREB+CBP can modestly, but significantly, activate transcription from sub-maximally chromatin assembled templates *in vitro*.

In order to assess the effect of CREB phosphorylation on HTLV-I transcription, the panel of additions was repeated, however, CREB was replaced with P-CREB, phosphorylated *in*

Figure 6.3: The effect of recombinant factors on transcription from sub-maximally assembled HTLV-I templates. Two independent experiments are shown. 300 ng of HTLV-I was assembled using the NAP-1 assembly system with half the amount of H3-H4 used in figure 6.2. DNA was assembled independently for both experiments. Recombinant activator proteins were added with the components of the assembly reaction (figure 6.1, panel A). The assembled templates were transcribed *in vitro*, resolved on a sequencing gel and exposed to a phosphoimage screen. The intensity of the bands were quantitated using image gauge software and are presented above the figure as phosphoimager (PI) units. Each lane contains 300 ng HTLV-I template, 1.3 ng NAP-1, 1.5 µl H2A/H2B and 1 µl H3/H4. The concentrations of the activators were 4 µg CREB or P-CREB, 5 µg Tax and 100 ng CBP. The proteins were added to the assembly reactions in the combinations indicated under the figure.


PI units
1200
1000
800
600
400
200
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
No additions
CREB
Tax
CREB+Tax
CBP
CREB+CBP
Tax+CBP
CREB+Tax+CBP
No additions
P-CREB
Tax
P-CREB+Tax
CBP
P-CREB+CBP
Tax+CBP
P-CREB+Tax+CBP
Naked DNA

vitro with PKA according to the methods (section 2.3.1.1a). Figure 6.3 shows that similar to CREB and CREB+Tax, P-CREB and P-CREB+Tax activated transcription compared to the basal level of transcription (compare lanes 10 and 12 with lanes 1 and 9 and lanes 2 and 4). However, P-CREB+CBP activated transcription to a greater degree than CREB+CBP (compare lanes 14 and 6). Surprisingly, unlike CREB+Tax+CBP, P-CREB+Tax+CBP did not result in transcriptional activation (compare lanes 8 and 16). Therefore activation of transcription from HTLV-I template requires CREB+Tax+CBP or P-CREB+CBP. Importantly, this experiment was repeated three times with different protein preparations to ensure the reproducibility of the data. Two independent experiments are shown in figure 6.3. It is also worth pointing out that since under mock assembly conditions, no activation of transcription is observed with these factors (see section 6.2.1; figure 6.2), this activation is from chromatin

The assembly reaction performed for the *in vitro* transcription reaction presented in figure 6.3 contained acetyl-coA to provide the substrate for any acetylation activity by the added activators. To determine if acetylation contributed to the transcriptional activation seen in figure 4.3, we performed a similar experiment with and without the addition of acetyl-coA . Figure 6.4 shows that acetyl-coA has no effect on the activation of HTLV-I transcription by the factors Tax, CREB and CBP. Therefore, we conclude that acetylation is not required for the modest activation of HTLV-I transcription seen on sub-maximally assembled templates.

6.2.2.2 Somatostatin templates

As a control to for the activity of the recombinant factors in the *in vitro* chromatin assembly and transcription system used in this work, the somatostatin promoter was used as a template for *in vitro* transcription. Like the HTLV-I promoter, the somatostatin template was assembled at sub-optimum levels with half the concentration of (H3,H4) required for maximum assembly (section 5.2.4, figure 5.13). Figure 6.5 shows that assembly at this level resulted in the repression (at least 20-fold) of transcription (lanes 1 and 9) compared to naked DNA (lane 17). Surprisingly, repression was more efficient using the somatostatin plasmid template. One possible explanation for this observation is that the somatostatin template is 2.7 Kb compared with the 3.4 Kb HTLV-I plasmid template. Smaller templates are known to be more efficiently assembled by *in vitro* chromatin assembly systems (Tremethick *et al.*, 1999)

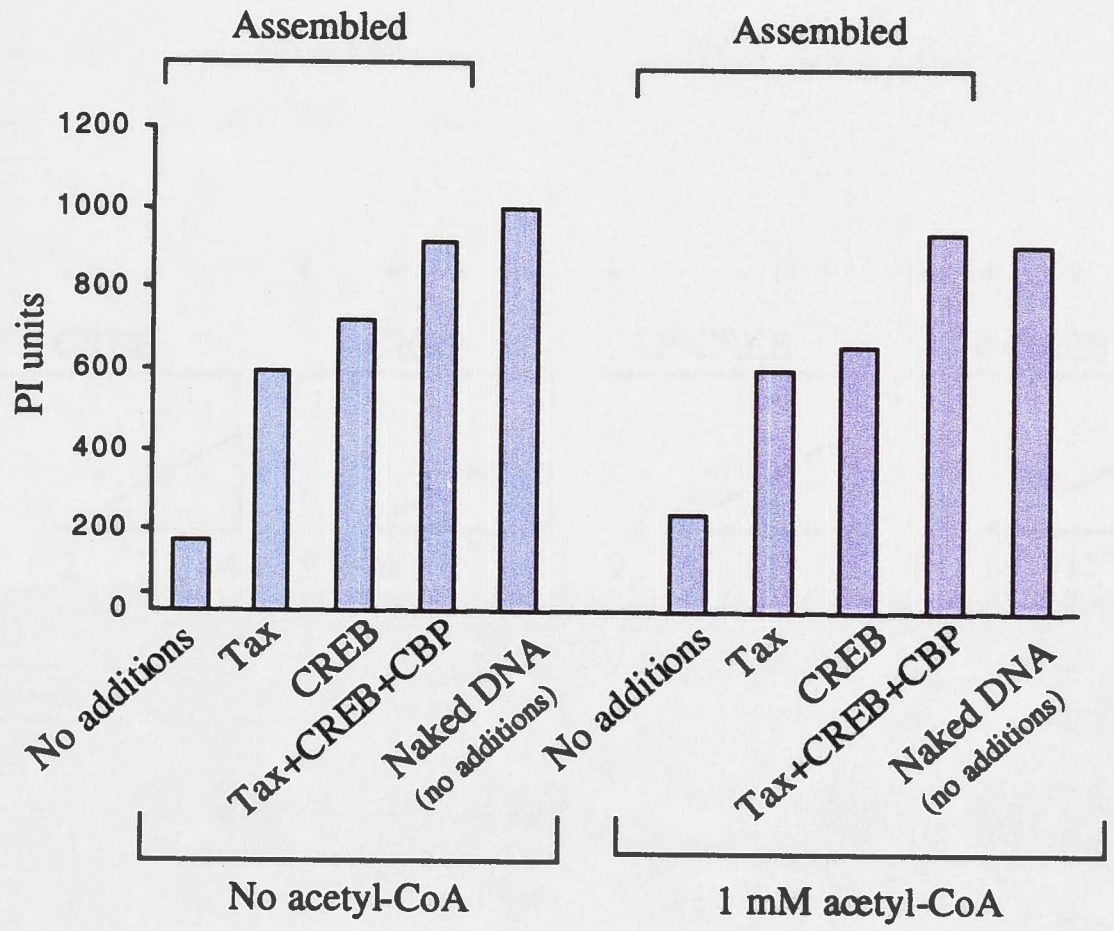


Figure 6.4: acetyl-coA has no effect on the levels of HTLV-I transcription on chromatin templates. 300 ng of HTLV-I DNA was assembled or mock assembled in the presence or absence of 1 mM acetyl-coA. Recombinant proteins were added with the components of the assembly reaction. The assembled templates were transcribed *in vitro* and resolved on a sequencing gel. The intensity of the bands was quantitated by phosphoimage analysis and are graphed. Each reaction (except for naked DNA) contained 300 ng HTLV-I template, 1.3 μg NAP-1, 1.5 μl H2A/H2B and 1 μl H3-H4. The concentrations of the activator proteins were 4 μg CREB or P-CREB, 5 μg Tax and 100 ng CBP. The proteins were added to the assembly reactions in the combinations indicated under the figure.

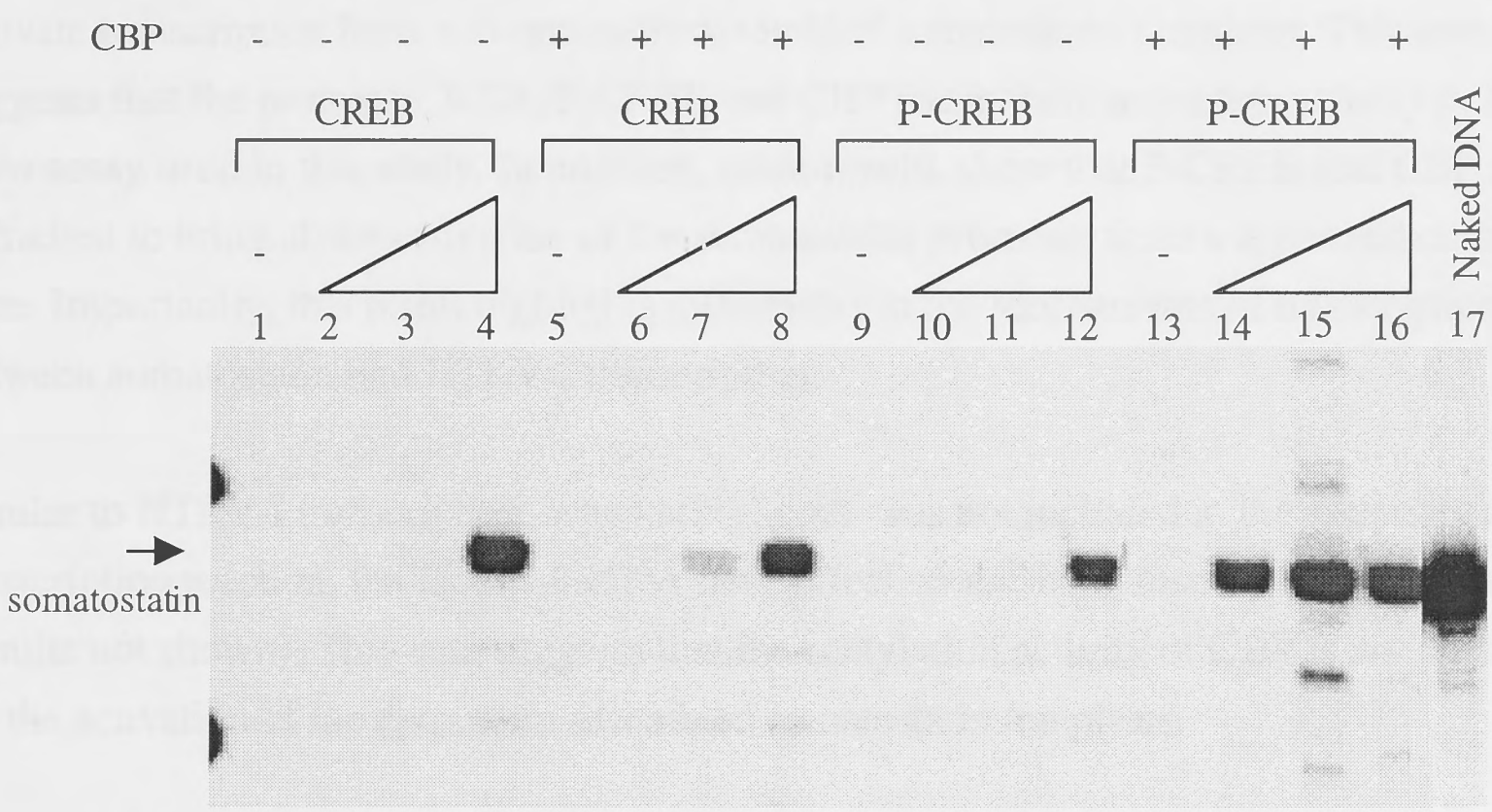


Figure 6.5: The effect of recombinant factors on transcription from sub-maximally assembled somatostatin templates. 300 ng of somatostatin DNA was assembled using the NAP-1 assembly system using half the amount of H3-H4 shown in figure 6.2 (panel B). The assembled templates were transcribed *in vitro* and resolved on a sequencing gel. Each lane [except naked DNA (lane 17)] contains 300 ng somatostatin template, 1.3 μg NAP-1, 1.5 μl H2A/H2B, 1 μl H3-H4. The concentrations of the recombinant proteins were 100 ng of CBP (lanes 5-8 and 13-16). Either 1 μg (lanes 2 and 6), 2 μg (lanes 3 and 7,) or 4 μg (4 and 8) CREB or 1 μg (lanes 10 and 14) 2 μg (lanes 11 and 15) or 4 μg (lanes 12 and 16) P-CREB. Lane 17: Naked DNA (mock assembled) with no additions.

Titration of CREB into the somatostatin assembly reaction resulted in an activation of transcription (compare lanes 4 and 1). Similarly, addition of P-CREB also activated transcription from the somatostatin template at the same concentration as CREB (compare lanes 9-12 with lanes 1-4). CREB, in combination with CBP was able to activate transcription at a lower concentration of CREB (compare lanes 5-8 with lanes 1-4). Thus, CREB+CBP is marginally more efficient at activating transcription in comparison to CREB alone. However, when P-CREB was added to the assembly reaction in combination with CBP, transcription was activated from the somatostatin template even at the lowest concentration of CREB (lanes 13-16). Therefore, exogenous P-CREB+CBP is able to activate transcription from sub-optimally assembled somatostatin templates. This result suggests that the proteins CREB, P-CREB and CBP retain their activation activity in the *in vitro* assay used in this study. In addition, these results show that P-CREB and CBP are sufficient to bring about activation of the somatostatin promoter from a repressed chromatin state. Importantly, this result highlights differences in the mechanisms of transcription between somatostatin and HTLV-I transcription.

Similar to HTLV-I transcription, when acetyl-coA was not included in the assembly and transcription reaction, the factors had the same effect on and somatostatin transcription (results not shown). This data suggests that the acetylation activity of CBP is not required for the activation of the chromatin assembled somatostatin templates.

6.2.3 A possible role for HMG-I(Y) in HTLV-I transcription control

Using the highly purified NAP-1 nucleosome assembly system, the results described in section 6.2.1 and 6.2.2 suggest that Tax, CREB and CBP are not sufficient for high level HTLV-I transcription. Although Tax, CREB and CBP may be necessary for transcriptional activation, these results suggest that other factors are involved to achieve high level transcription on chromatin assembled templates.

6.2.3.1 Interaction of HMG-I(Y) with the HTLV-I LTR

Work from this laboratory has shown that the high mobility group (HMG) protein HMG-I(Y) may be an important factor for HIV-I viral expression (Henderson *et al.*, 2000) (section 1.2.2). In work by these investigators, *in vitro* gel mobility shift assays using the non-specific DNA competitor poly (dG.dC) demonstrate that HMG-I(Y) binds specifically to the HIV-I promoter. This binding by HMG-I(Y) may not have been detected by other

investigators as the commonly used non-specific DNA competitor poly (dA.dT) also binds HMG-I(Y) preventing its binding to the HIV promoter in gel shift assays (Henderson *et al.*, 2000).

To assess whether HMG-I(Y) can bind to the HTLV-I LTR, HMG-I was purified from human placenta according to the methods (section 2.3.1.4). Figure 6.6 shows a Coomassie blue stained SDS-PAGE of purified HMG-I. We estimate that the HMG-I preparation is 95% pure. To determine the exact number of HMG-I binding sites, and the exact binding positions relative to the HTLV-I LTR, the binding of increasing concentrations of HMG-I was analysed by partial DNase I digestion. Figure 6.7 (panel A) demonstrates that three regions of the 147 bp HTLV-I LTR are protected from partial DNase I digestion by HMG-I. This protection pattern was confirmed by DNase I digestion of the reverse strand (panel B). The position of the protected areas in relation to the major features of the HTLV-I promoter are shown in figure 6.8. HMG-I(Y) footprint regions 2 and 3 comprise the classical A/T-rich binding sequences shown to be associated with HMG-(I) binding through the AT hook (Reeves and Nissen, 1990). However, site 1 consists of an unusual binding site (TCCCCATGT). As the HMG-I preparation is at least 95% pure, and HMG footprint region 1 has been confirmed on both positive and negative strands it is likely that region 1 is created by protection of the DNA by HMG-I. Footprint region 3 may represent the ability of HMG-I to bind to structural elements of the DNA rather than specific sequence.

6.2.3.2 Function of HMG-I(Y) binding *in vitro*

Since HMG-I(Y) can bind *in vitro* to the HTLV-I LTR, we began a preliminary investigation into the functional significance of this binding. Work in this laboratory has demonstrated that HMG-I(Y) can affect the binding of AP-I family members on the HIV-I promoter (Henderson *et al.*, 2000). Furthermore, HMG-I(Y) is known to bind preferentially to bent DNA and in addition, bend DNA upon binding (Reeves and Nissen, 1990). Significantly, CREB is also thought to bind differentially to DNA with differing bend angles (Yin *et al.*, 1996). Therefore, the simultaneous binding of HMG-I and CREB to the HTLV-I 147 bp promoter and the 180 bp somatostatin promoter was investigated. Figure 6.9 demonstrates that binding of HMG-I to the HTLV-I LTR results in a gel mobility shift characteristic of high affinity HMG-I binding (Henderson *et al.*, 2000) (lanes 2-4). HMG-I binds to the somatostatin probe with lower affinity and may relate to the non-specific DNA binding properties of HMG-I (lanes 10-12). Binding of both CREB and

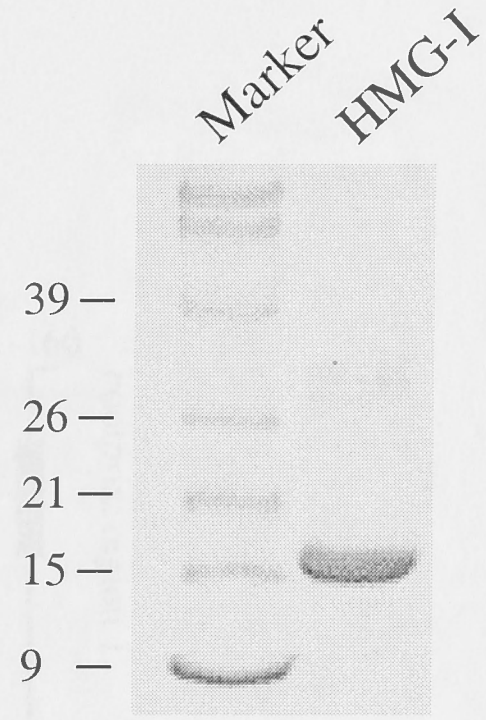


Figure 6.6. Purification of HMG-I from human placenta.
Coomassie brilliant blue stained 15% SDS-PAGE of HMG-I purified from human placenta. Molecular weight markers (kDa) are shown on the left.

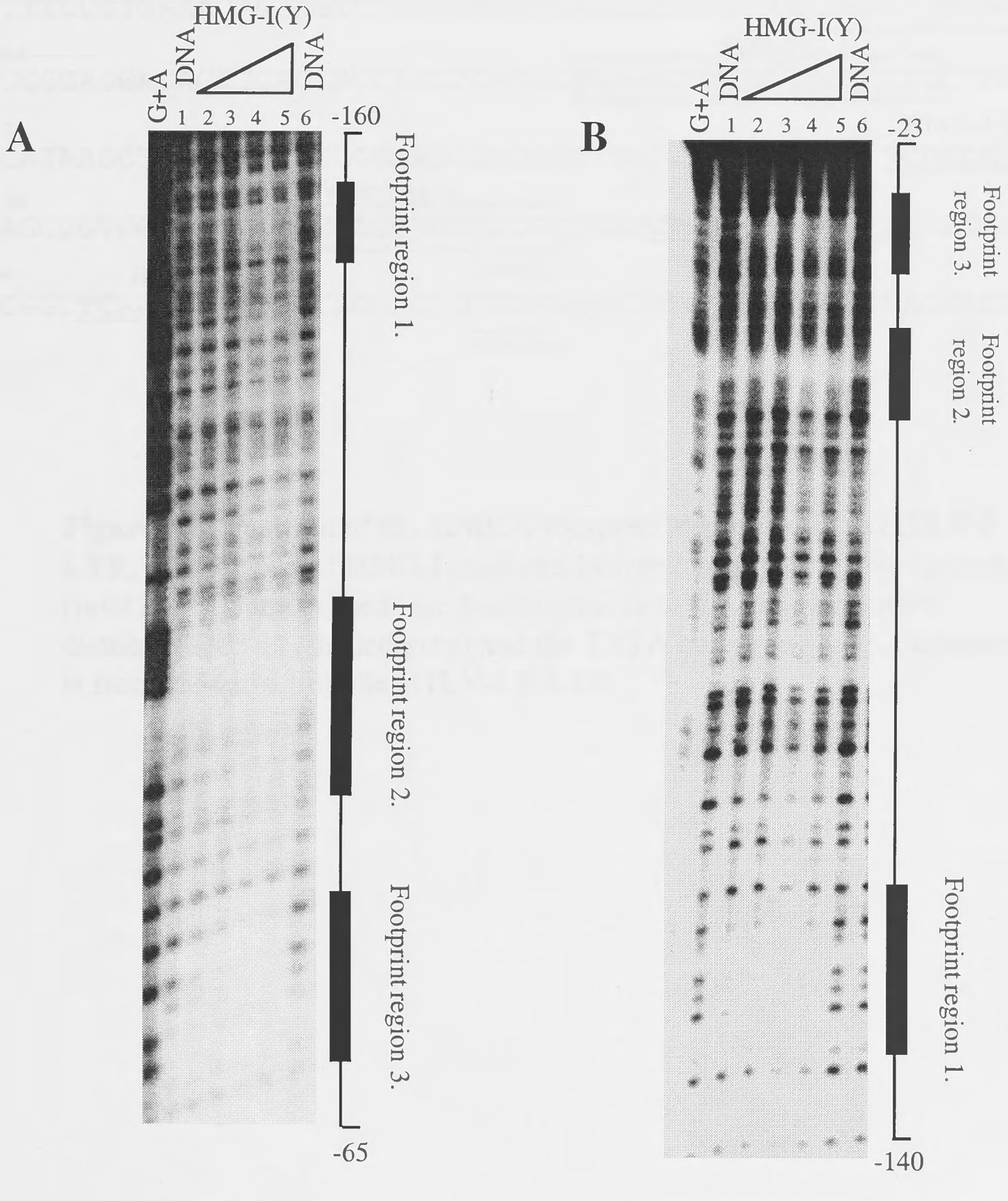


Figure 6.7: DNase I footprinting analysis of HMG-I binding to the HTLV-I promoter. Increasing concentrations of HMG-I from figure 6.6 was bound to the 147(M) probe (spanning region -166 to -26) on the HTLV-LTR. Panel A shows the forward (sense) strand and panel B shows the reverse (anti-sense) strand. The probe was partially digested with 0.5 U (HMG-I bound) or 0.1 U (naked DNA) DNase I and resolved on a sequencing gel. Lanes 1 and 6: Naked DNA; lanes 2-5: 50, 100, 200 and 400 ng HMG-I. Regions of DNase I protection are labelled region 1, 2 and 3 respectively. G+A ladders are shown on the left of each panel.

Figure 6.8: Position of the HMG-I footprint regions on the HTLV-I LTR. The regions of HMG-I mediated DNase I protection from figure 6.7 (bold text and underlined) are shown relative to the Tax responsive element (TRE-1) (shaded grey) and the TATA box (open box). Sequence is from -354 to +1 on the HTLV-I 3' LTR.

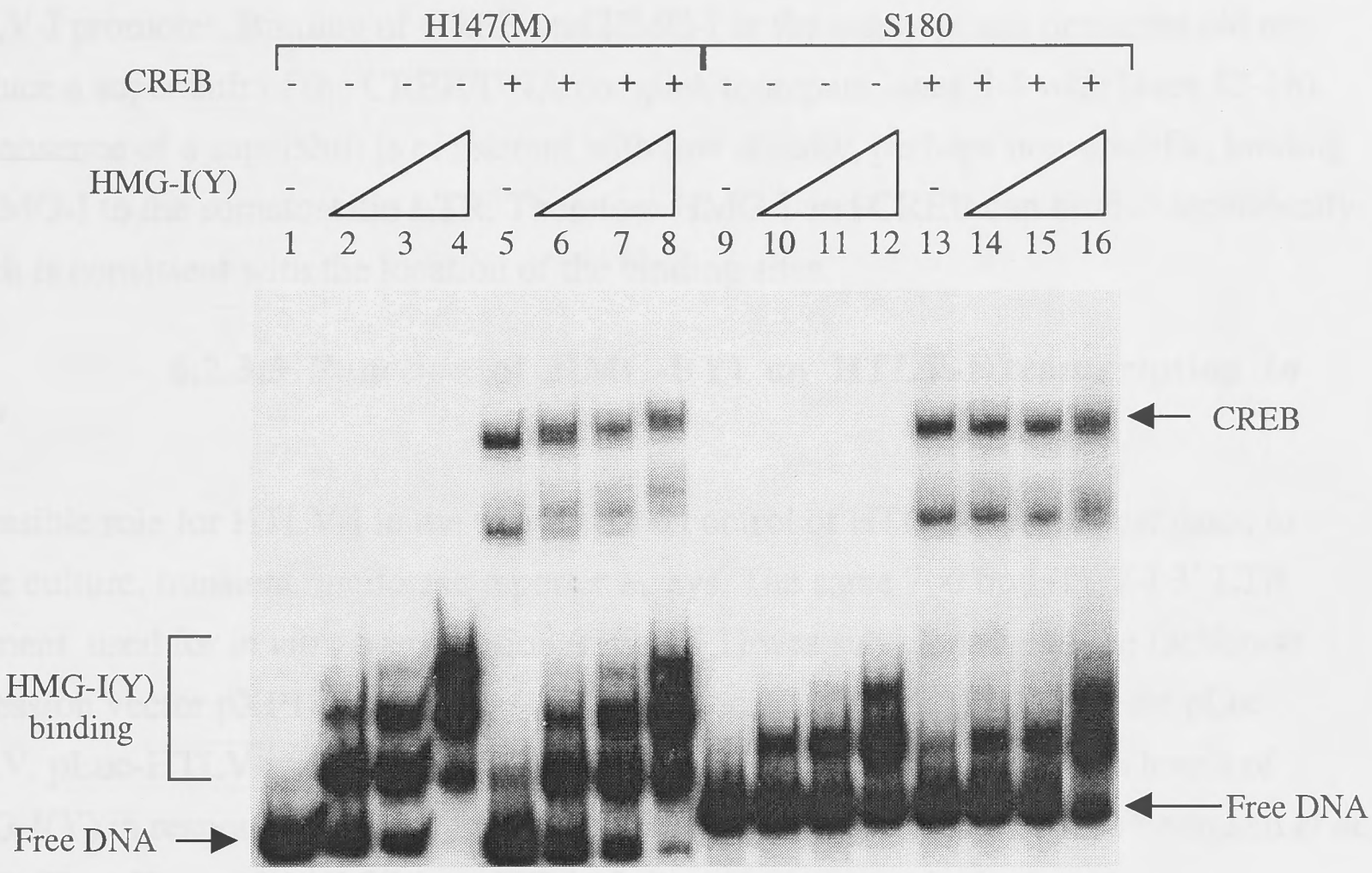


Figure 6.9: Binding of HMG-I to the HTLV-I LTR has no effect on the binding affinity of CREB. Gel mobility shift assay showing increasing concentrations of HMG-I bound to the HTLV-I H147 probe (lanes 1-8) or the somatostatin S180 probe (lanes 9-16) with or without 2 μg CREB. Lanes 2-4 and 10-12: 5, 10, and 20 ng HMG-I(Y). Lanes 6-8 and 14-16: 2 μg CREB and 2, 4 and 25 ng HMG-I. HMG-I/DNA complexes are indicated by a bracket. Free DNA and CREB/DNA complexes are indicated by an arrow.

HMG-I to the HTLV-I probe resulted in a supershift of the CREB DNA complex (lanes 5-8) suggesting that HMG-I and CREB can occupy their binding site on the HTLV-I LTR simultaneously. In addition, HMG-I had no effect on the binding affinity of CREB to the HTLV-I promoter. Binding of CREB and HMG-I to the somatostatin promoter did not produce a supershift of the CREB/DNA complex (compare lanes 5-8 with lanes 13-16). The absence of a supershift is consistent with low affinity, perhaps non-specific, binding of HMG-I to the somatostatin LTR. Therefore HMG-I and CREB can bind independently which is consistent with the location of the binding sites.

6.2.3.3 Function of HMG-I(Y) on HTLV-I transcription *in vivo*

A possible role for HTLV-I in the transcription control of HTLV-I was investigated in tissue culture, transient, luciferase reporter assays. The same 700 bp HTLV-I 3' LTR fragment used for *in vitro* transcription (figure 5.1) was sub-cloned into the luciferase expression vector pXP1-G according to the methods (section 2.12.1) to create pLuc-HTLV. pLuc-HTLV was transfected into Jurkat T-cells which express high levels of HMG-I(Y) in response to stimulation with PMA (Henderson *et al.*, 2000; Friedmann *et al.*, 1993). The effect of HMG-I(Y) on HTLV-I transcription was determined by co-transfection of the antisense RNA HMG-I(Y) construct (pRcCMVIGMH) with pLuc-HTLV (section 2.12).

Three independent transfection experiments are shown in figure 6.10. Panel A shows that co-transfection of antisense HMG-I(Y) with pLuc-HTLV inhibited HTLV-I transcription in a dose dependent manner with a maximum reduction in transcription of 2.6-fold in cells stimulated with PMA. Co-transfection of 1μg of the Tax expression plasmid BC15S (section 2.12.1) with pLuc-HTLV-I activated the expression from the HTLV-I promoter in Jurkat cells induced with PMA (panel B). Co-transfection of anti-sense HMG-I(Y) resulted in only a small reduction of HTLV-I transcription (1.6 fold) of Tax activated transcription in PMA treated cells. Figure 6.10, (panel C) shows that anti-sense HMG-I has no effect on the Tax mediated activation of unstimulated cells. In addition, anti-sense HMG-I had no effect on the levels of HTLV-I transcription from the HTLV-I reporter when the Jurkat cells were not stimulated with PMA (results not shown). Anti-sense HMG-I(Y) may have had no effect on the transcription from the HTLV-I reporter in non-stimulated cells as PMA stimulation is required to induce HMG-I expression in Jurkat cells (Henderson *et al.*, 2000; Friedmann *et al.*, 1993). Moreover, as anti-sense HMG-I(Y) had only a small effect on

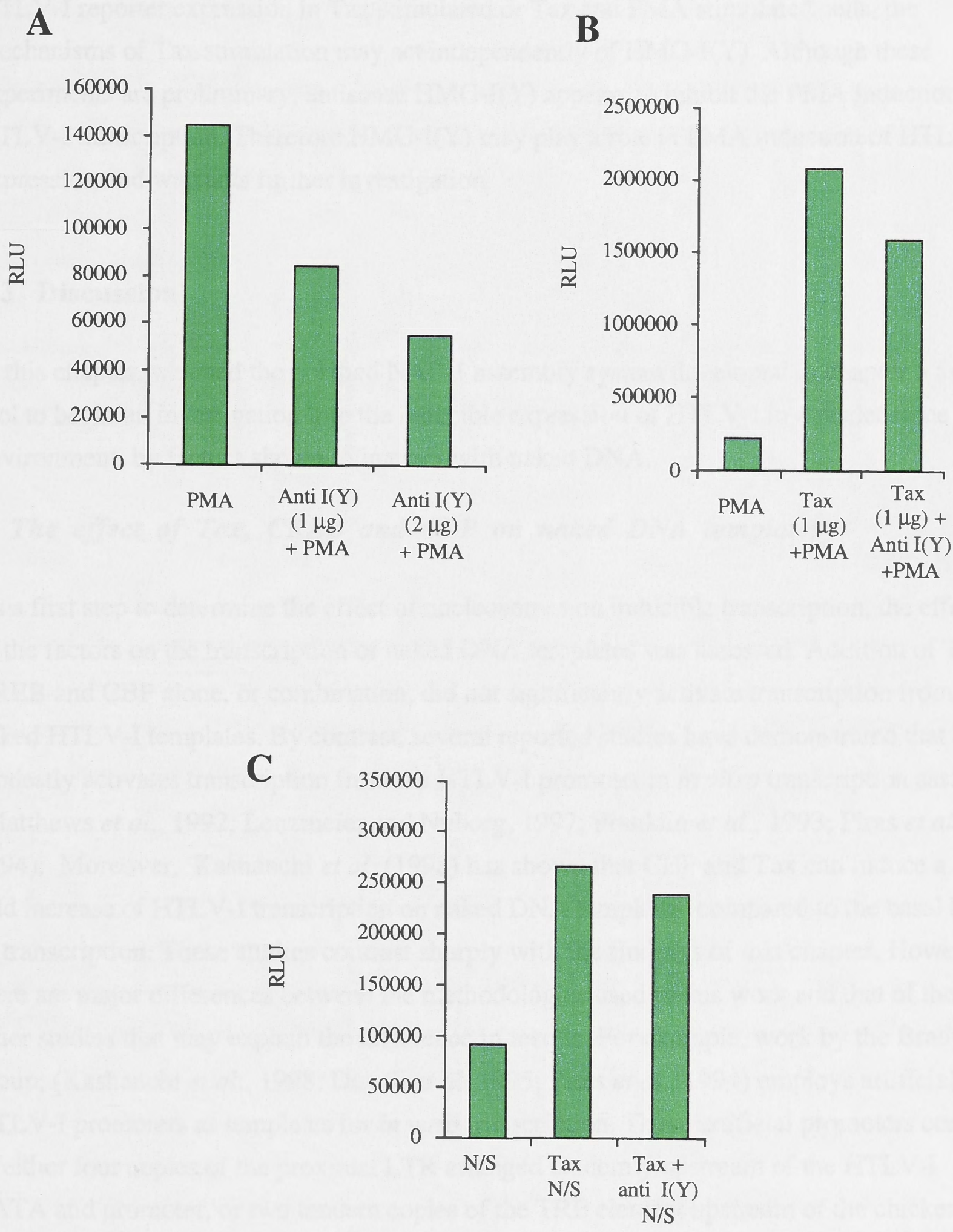


Figure 6.10: Antisense HMG-I can inhibit PMA induced transcription in transient transfection assays. A, **B** and **C** represent 3 independent transfection experiments. Jurkat T cells were transfected with 1 µg pLuc-HTLV luciferase reporter construct alone or co-transfected with anti-sense HMG-I (pRcCMVIGMH) (1 µg unless indicated) and/or the Tax expression plasmid (BC15s) (1µg unless indicated). Cells were stimulated with 32 nM PMA for 9 hr where indicated. Expression from pLuc-HTLV was determined by the luciferase assay. Values for the luciferase light reaction are shown as relative light units (RLU).

HTLV-I reporter expression in Tax stimulated or Tax and PMA stimulated cells, the mechanisms of Tax stimulation may act independently of HMG-I(Y). Although these experiments are preliminary, antisense HMG-I(Y) appears to inhibit the PMA induction of HTLV-I transcription. Therefore HMG-I(Y) may play a role in PMA induction of HTLV-I expression and warrants further investigation.

6.3 Discussion

In this chapter, we used the purified NAP-1 assembly system developed in chapter 5 as a tool to begin an investigation into the inducible expression of HTLV-I in a nucleosome environment, by factors shown to interact with naked DNA.

a. The effect of Tax, CREB and CBP on naked DNA templates

As a first step to determine the effect of nucleosomes on inducible transcription, the effect of the factors on the transcription of naked DNA templates was assessed. Addition of Tax, CREB and CBP alone, or combination, did not significantly activate transcription from naked HTLV-I templates. By contrast, several reported studies have demonstrated that Tax modestly activates transcription from the HTLV-I promoter in *in vitro* transcription assays (Matthews *et al.*, 1992; Lenzmeier and Nyborg, 1997; Franklin *et al.*, 1993; Piras *et al.*, 1994). Moreover, Kashanchi *et al.* (1998) has shown that CBP and Tax can induce a 30 fold increase of HTLV-I transcription on naked DNA templates compared to the basal level of transcription. These studies contrast sharply with the findings of this chapter. However, there are major differences between the methodologies used in this work and that of the other studies that may explain the difference in results. For example, work by the Brady group, (Kashanchi *et al.*, 1998; Duvall *et al*, 1995; Piras *et al.*, 1994) employs artificial HTLV-I promoters as templates for *in vitro* transcription. These artificial promoters consist of either four copies of the proximal LTR arranged tandemly upstream of the HTLV-I TATA and promoter, or two tandem copies of the TRE element upstream of the chicken ovalbumin TATA box. Tandem arrangements of multiple copies of the GAL4 enhancer element have been shown to increase factor binding and transcription levels (Steger *et al.*, 1997; Laybourn and Kadonaga, 1992). In addition, basal and inducible transcription of HTLV-I is dependent upon the number of TRE elements and their spacing relative to each other (Goren *et al.*, 1999). Such arrangement of TRE elements may also alter the responsiveness of the promoter to Tax, CREB and CBP.

b. Transcription from maximally assembled nucleosomal templates

The second area of investigation employed in this chapter was to examine the effect of Tax, CREB and CBP on HTLV-I promoter templates maximally assembled into nucleosomes. When the HTLV-I promoter was assembled into chromatin at a high nucleosome density by the NAP-1 assembly system, the addition of exogenous Tax and CBP at the beginning of the assembly reaction was not sufficient to activate transcription. Moreover, maximum assembly of the somatostatin promoter by the purified NAP-1 assembly system was also refractory to transcription by CREB and P-CREB alone or in combination with CBP. Therefore, at high nucleosome densities, nucleosomes could displace CREB, perhaps at the level of the nucleosome. Alternatively, given that CREB can bind efficiently to a single nucleosome (chapter 4), folding of the chromatin fibre into a condensed structure may disrupt CREB-CBP interactions or any other functional interactions that lead to transcriptional initiation. Electron microscopic studies have shown that with increasing nucleosome density, plasmid DNA is folded into more tightly compacted structures (Shimamura *et al.*, 1988). These results suggest that additional factors are required to overcome the repressive effect of fully compacted chromatin. That is, Tax+CBP may be necessary but not sufficient for transcriptional activation of HTLV-I in this condensed chromatin environment.

It is important to note however, that the NAP-1 system of assembly uses purified components to assemble nucleosomes. The use of a purified system of chromatin assembly was necessary to create a repressive chromatin environment (chapter 5) and to remove potential complications with regard to interpreting the data such as the presence of chromatin remodelling factors. Therefore, the promoter templates were assembled in the absence of factors naturally found in chromatin *in vivo*. Such proteins include the linker histones H1 and variants, non-histone proteins such as the HMG family and natural ATP dependent spacing activities. Thus, the nucleosome arrays generated by the NAP-1 assembly system may be more densely assembled causing an impenetrable block to inducible transcription by the factors used in this study. This notion is supported by work with the *Drosophila* S-190 chromatin assembly system which demonstrates that the presence of ATP-utilising nucleosome remodelling factors in the S-190 extract are important for transcription of the chromatin templates (Kraus and Kadonaga, 1998; Pazin *et al.*, 1998; Mizuguchi *et al.*, 1997; Pazin *et al.*, 1994). Furthermore, addition of HMG 17 to assembly reactions has been shown increase inducible transcription with S-190 assembled templates (Kraus and Kadonaga, 1998; Paranjape *et al.*, 1995). HMG 14 and 17 have also

been shown to space nucleosomes assembled with the *Xenopus* N1/N2(H3,H4) system and potentiate transcription form chromatin templates (Tremethick, 1994; Tremethick and Drew, 1993). Moreover, in chapter 5 of this thesis, we identified an activity in the N1/N2(H3,H4) complex that activated transcription from HTLV-I assembled templates (section 5.2.3). Although maximum assembly by NAP-1 may represent artificially high level of nucleosome assembly, this system can be used as a tool to identify cellular factors which may enable transcription from the assembled templates in combination with Tax, CREB and CBP. This could be accomplished by the sequential addition of factors and complexes known to be involved in chromatin remodelling and transcriptional activation.

c. Transcription from sub-maximally assembled templates

Although maximally assembled chromatin templates are refractory to inducible transcription by factors tested in this study, reducing the concentration of the (H3,H4) tetramer enabled inducible transcription from HTLV-I templates. Reduction in the level of (H3,H4) results in the less dense assembly of all plasmids in the preparation rather than the full assembly of a proportion of plasmids leaving some plasmids as naked DNA (Tremethick, 1999). Therefore the transcription detected results from transcription from less densely assembled nucleosomes and not naked DNA. Templates with a lower density of nucleosomes can still repress transcription (100 fold compared to 10 fold) but this lower density would result in less compact structures and less chance of nucleosome interference with activation complex formation. Furthermore, chromatin remodellers have been shown to be involved in the process of nucleosome sliding (Hamiche *et al.*, 1999). Lower nucleosome density would result in the nucleosomes being more fluid. Thus, lower nucleosome density may mimic the presence of such chromatin remodelling proteins.

On the less densely assembled HTLV-I templates, Tax+CREB+CBP and P-CREB+CBP activated transcription, whereas CBP alone had a small effect on transcription. These results suggest that activation of HTLV-I transcription by CBP is dependent upon recruitment of CBP to the HTLV-I promoter by Tax+CREB or P-CREB. Addition of Tax alone to the transcription reaction also had a minor effect on transcription and is consistent with its requirement for CREB binding to target it to the promoter. These observation are consistent with the model we originally proposed for HTLV-I LTR activation (figure 1.12).

Surprisingly, addition of exogenous CREB alone, Tax+CREB and P-CREB+Tax were able to activate transcription from the sub-optimally assembled templates without the

requirement for CBP. It could be argued that endogenous CBP present in the Jurkat nuclear extract may enable this transcriptional activation to occur. However, this does not explain the ability of CREB to activate transcription as it is thought that phosphorylation of CREB is required for CBP recruitment. The concentration of CREB used in this study was empirically determined as the concentration that gave the maximum amount of transcriptional activation. Perhaps high levels of exogenously added CREB can bind to the viral promoter and activate transcription by mechanisms apart form the recruitment of CBP. For example, Ferreri *et al.*, 1994 have shown that a CREB mutant, lacking the kinase inducible domain (site of phosphorylation) continues to act as a potent constitutive activator *in vitro*. These authors demonstrate that this mutant CREB interacts with components of the TFIID transcription complex though its Q2 domain (see figure 1.11, section 1.8.2.1).

A second somewhat unexpected result was that P-CREB+Tax+CBP was unable to activate transcription from the nucleosome assembled templates. One possibility could be that Tax may directly interact with CBP unbound to DNA (Kwok *et al.*, 1996). Thus, Tax may squelch CBP, ie, prevent the binding of CBP to the promoter. In support of this possibility, Lenzmeier *et al.* (1998) has demonstrated that Tax requires direct access to DNA for recruitment of CBP to the promoter. Furthermore, competition for limiting CBP has been proposed as a mechanisms for the observed repression of AP-1 transcription activity by nuclear hormone receptors (Kamei *et al.*, 1996). Moreover, Van Orden *et al.*, (1999) demonstrated that Tax competes with c-Jun for its binding site on CBP (see figure 1.7) and represses transcriptional functions of CBP *in vivo*.

In this study, CREB, P-CREB and P-CREB+CBP were able to activate transcription in the absence of Tax suggesting that the activation domains of CREB and/or CBP are required for activation within the nucleosomal environment. Since Tax+CBP had no effect on HTLV-I transcription in this study, it appears that CREB (or P-CREB) is critical for activation by the Tax+CREB+CBP activation complex. The ability of CREB to significantly overcome repression is consistent with it ability to bind a single nucleosome as demonstrated in chapter 4.

d. The role of CBP and acetylation in HTLV-I transcription on sub-maximally assembled templates

Although CBP has been shown to interact with Tax *in vitro*, there is a lack of evidence in the literature that directly implicates CBP as a necessary factor for HTLV-I transcription

and the mechanism by which CBP activates transcription is unclear. In this study, CREB+Tax+CBP activated transcription to the greatest degree of the combinations of factors tested in this study. However, addition of CBP only modestly activated transcription over the levels detected for CREB, CREB+Tax and P-CREB+Tax. It has been suggested that the level of CBP in cells may be limiting (Petrij *et al.*, 1995). If CBP is necessary for transcriptional activation then, exogenously added CBP should result in a substantial increase in transcriptional activation.

One way in which CBP is thought to activate transcription is through its ability to acetylate histones. Indeed, Kraus and Kadonaga have argued that transcriptional activation through p/300 occurs only in the context of chromatin. However, the level of factor mediated transcriptional activation on the nucleosomal templates in the presence of acetyl-coA is the same as in the absence of acetyl-coA . This observation suggests that the acetylation activities of CBP are not required for the activation of transcription in this purified nucleosome system. Several other studies have shown the CBP can activate gene expression independently of acetylation. Such studies include Kashanchi *et al.* (1998) who showed activation of the naked HTLV-I promoter by CBP in the absence of acetyl-coA. Furthermore, Myall *et al.* (1997) showed CBP mediated activation from the TCRα enhancer assembled into chromatin independently of acetyl-coA.

Several recent studies have suggested that Tax+CREB+CBP may not be sufficient for high level transcriptional activation of the HTLV-I LTR. For example, biochemical analysis of a well characterised Tax mutant M47, indicate that it retains the ability to interact with CREB, 21-bp repeats, and CBP, yet remains comparatively defective in transactivation of the HTLV-I LTR (Harrod *et al.*, 1998; Smith and Greene, 1990; Tie *et al.*, (1996). These results suggest that recruitment of CBP to the Tax+CREB+TRE complex is not sufficient for Tax dependent trans-activation. Thus, interactions between Tax and additional cellular co-activators or general transcription factors are likely to be essential. Furthermore, a recent report by Harrod *et al.* (2000) has demonstrated that Tax must interact with the p300/CBP associated factor (p/CAF), as well as CBP in order to achieve maximum levels of transcription. Therefore, it would be interesting to incorporate purified, recombinant p/CAF into the reconstitution reactions developed in this study. Since these reconstitution reactions employ purified components, this system would be valuable in determining whether p/CAF recruitment to the Tax+CREB+CBP complex is sufficient for high level transcriptional activation.

This study represents the first step of using the NAP-1 system to evaluate the transcriptional control of HTLV-I. However is important to note that the results generated regarding the effect of Tax, CREB and CBP on HTLV-I transcription should be interpreted with some degree of caution for several reasons. Firstly, the factors were added to the assembly system with the components of the assembly system and this may not accurately represent the situation *in vivo* where these factors may interact with pre-assembled templates. Although, the NAP-1 assembly system developed in this work could be used for this purpose in future studies. It would also be interesting to pre-bind factors to DNA in various orders of addition to overcome potential competition problems. Secondly, although the experiments presented in this chapter were repeated at least twice to ensure reproducibility, the transcription reaction is highly dependent upon the level of supercoiling or assembly of the template DNA. Using the NAP-1 assembly system, the degree of assembly is dependent upon the ratios of the components and slight variation in the concentration of each of the components may produce a significant amount of variation in the level of repression and transcription. Thirdly, even though the NAP-1 system is a purified assembly strategy, the possibility that NAP-1 itself interacts with the factors influencing transcription cannot be discounted. For example Walter *et al.* (1995) has shown that NAP-1 can stimulate GAL4-AH binding and nucleosome displacement. In addition NAP-1 has been shown to be a functional homologue of TAF-1, a factor that is required for transcription of chromatin templates (Okuwaki *et al.*, 1996).

In this study, the transcriptional activation detected for HTLV-I by CREB+Tax+CBP was modest (maximum of 8 fold) in comparison with the 30 fold activation of HTLV-I transcription by CBP+Tax on naked DNA templates (Kashanchi *et al*, 1998). Significant differences in promoter template used by Kashanchi *et al.* (1998) and the present work may partly explain the differences in transcription activation as previously discussed. However, the high levels of transcription detected for HTLV-I nucleosomal templates in the presence of the *Xenopus* S-150 and N1/N2(H3,H4) assembly extracts in chapter 5 (section 5.2.3), indicate that HTLV-I can be expressed at high levels within a chromatin environment. The modest transcription detected from sub-optimally nucleosome assembled HTLV-I templates is not likely to account for the high level of activation of viral transcription preceding viral replication and disease. Therefore we conclude that CREB, Tax and CBP are not sufficient for high level transcriptional activation of HTLV-I.

It could be argued that the inability of the recombinant factors to activate transcription on fully assembled templates and only modestly activate transcription on sub-optimally assembled templates, could be due to the activity of the recombinant factors. For example, the recombinant factors may have lost either part, or all of the transcriptional activity in the purification process. However, chapter 1 of this thesis demonstrated that CREB and Tax exhibited functional characteristics consistent with published studies. Chapter 1 also demonstrated that recombinant CBP retained its ability to acetylate histones and other factors. In this study, the use of somatostatin as a control template for CREB mediated transcription shows that a combination of P-CREB and CBP was required to activate transcription. This observation supports the findings of chapter 1 that the recombinant proteins have retained their activity throughout the purification process.

e. Transcription from somatostatin templates

Like the HTLV-I promoter, the maximally assembled somatostatin template was refractory to activation by the exogenous addition of CREB and P-CREB alone or with CBP. However, on the somatostatin promoter assembled with half the amount of (H3,H4) tetramer, CREB and P-CREB activated transcription from the repressed state. Furthermore, while CBP alone had no effect on transcription, addition of CBP and P-CREB markedly activated transcription even at the lowest level of P-CREB (see figure 6.5). This result is consistent with the model for CBP activation where CREB acts to recruit CBP to the promoter. The ability of CREB and CBP to activate less densely assembled chromatin templates is consistent with the possibility that de-repression may require other factors such as chromatin remodellers or spacing activities that may mimic a more open chromatin conformation obtained using lower levels of (H3/H4). As was the case for HTLV-I, the acetylation activity of CBP was not required for activation as activation occurred without acetyl-coA. Other functions of CBP besides acetylation such as interactions with the basal transcriptional machinery, may explain the ability of CBP to activate somatostatin expression on chromatin templates.

f. A possible role for HMG-I(Y) in HTLV-I transcription

Since one major finding of this chapter demonstrates that Tax+CREB+CBP is not sufficient for activation using highly condensed chromatin templates, we began a preliminary investigation into a possible role for HMG-I(Y). HMG-I(Y) has a well defined role in the regulation of the INF-β enhanceosome and has recently been shown to be

involved in factor binding to the HIV-I retrovirus (Henderson *et al.,* 2000). In this chapter, we demonstrated that HMG-I(Y) can bind specifically to the HTLV-I promoter. In addition, the demonstration the anti-sense HMG-I(Y) inhibits PMA activated HTLV-I transcription in tissue culture reporter assays is consistent with a role for HMG-I(Y) in transcriptional control. This study represents the initial steps of a more comprehensive investigation required to characterise the role of HMG-I(Y) in HTLV-I expression. It would be useful to continue the *in vivo* tissue culture analysis in a chromatin context. This could be achieved by similar studies using cells stably transfected with the pLuc-HTLV-I plasmid or the use of mini-chromosomes. It would also be interesting to follow up this investigation by examining the effect of HMG-I(Y) in the context of the NAP-1 assembly system. HMG-I(Y) could be added to the assembly reactions alone or in combination with Tax, CREB and CBP to determine whether the binding of HMG-I(Y) to the HTLV-I LTR has a functional significance.

We have used a purified system of nucleosome assembly in this study to show that first, Tax, CREB and CBP are not sufficient for activation of HTLV-I templates maximally assembled into nucleosomes. In addition CREB, P-CREB and CBP are also not sufficient for transcriptional activation of the maximally assembled somatostatin promoter. Secondly, we showed that Tax+CREB+CBP can modestly activate transcription on sub-optimally assembled templates. This is the first demonstration of inducible transcription from the HTLV-I LTR on chromatin assembled templates *in vitro*. Moreover, P-CREB+CBP could activate transcription from sub-maximally assembled somatostatin templates. This result indicates that CBP may function differently on different promoters. Thirdly, we demonstrated that the acetylation activity of CBP was not necessary for the levels of activation seen in this study. Importantly, this study has endeavoured to closely resemble the *in vivo* situation by using full length proteins and natural promoter sequences. Taken together, the results of this chapter suggest that Tax+CREB+CBP is not sufficient for high level transcription of HTLV-I in a chromatin environment and implies that other factors must be recruited to the promoter to result in Tax mediated transcription. The development of the *in vitro* NAP-assembly and transcription system can now be employed to identify factors such as HMG-I(Y) that may be involved in the control of HTLV-I transcription *in vivo*.

CHAPTER 7:

General discussion

Significant achievements

To gain a better understanding of the role of chromatin in the regulation of transcription, we employed an *in vitro* biochemical approach using the HTLV-I promoter, and the factors that interact with it, as a model in which to investigate the relationship between nucleosome assembly and inducible transcription. The significant findings of this work include:

(a) CREB is able to interact with its recognition site when the site is incorporated into a nucleosome.

As an important first step in the investigations, we studied the ability of CREB to interact with its recognition site incorporated into a nucleosome. Strikingly, CREB binds to either a single high affinity or low affinity site with only an 8 fold reduction in affinity relative to naked DNA. Importantly, this binding resulted in the formation of a stable DNA/histone octamer/CREB complex. This represents the first demonstration of the ability of CREB to bind with high affinity to a pre-assembled nucleosome and establishes CREB as one of a set of a strong nucleosome binding factors. Furthermore, these findings indicate that chromatin remodelling complexes such as SAGA, SWI/SNF, NURF, CHRAC, or ACF, are not required for efficient binding of CREB to its recognition site on a nucleosome. This is significant with regards to the transcriptional regulation of HTLV-I since CREB binding to the TRE element of the LTR is a necessary first step for the recruitment of Tax *in vitro*, illustrating its vital role in subsequent HTLV-I specific activation complex formation (Kwok *et al.*, 1996; Lemzimer *et al.*, 1998). As a retrovirus, HTLV-I exists within the chromatin assembled environment of the host cell genome. Clearly, the demonstration that CREB binds with high affinity to a single nucleosome *in vitro* suggests that CREB binding to chromatin *in vivo* may be a vital step in HTLV-I viral gene expression. This finding is also consistent with the ability of CREB to overcome chromatin mediated repression [discussed in point (c)]

(b) The establishment of an *in vitro* nucleosome assembly system.

The end point of *in vitro* studies involving the interaction between transcription factors and chromatin is to determine whether a transcriptionally repressed chromatin template can be

activated to a transcriptionally permissive or active template. A major outcome of this research has been the establishment of a purified *in vitro* nucleosome assembly system, coupled to an *in vitro* transcription system, that can be used to examine inducible transcription of promoter templates organised into a nucleosome array. Several well characterised nucleosome assembly systems such as protein extracts from *Xenopus* oocytes and *Drosophila* embryos contain factors that modulate the transcription of chromatin. As a purified strategy, the NAP-1 system enables the direct effect of transcription factors on transcriptionally repressed nucleosomal templates to be examined, free from many of the contaminants present in the *Xenopus* and *Drosophila* preparations. Importantly, the NAP-1 system could be used to assemble the diverse promoters studied in many laboratories into nucleosomes. Assembly using the NAP-1 system may thereby allow the investigation of precise molecular mechanisms involved in factor induced de-repression and transcriptional activation in a nucleosome environment.

(c) Use of the NAP-1 assembly system to investigate the inducible transcription of HTLV-I in chromatin.

The NAP-1 system was employed in this study to begin an investigation into the ability of the HTLV-I interacting factors Tax, CREB and CBP to de-repress or activate expression from transcriptionally repressed templates. On fully assembled HTLV-I and somatostatin templates CREB, Tax and CBP could not de-repress transcription. However, Tax+CREB+CBP (HTLV-I template) and P-CREB+CBP (somatostatin template) could de-repress partially assembled chromatin templates. This result demonstrates that these factors can deal with the chromatin problem. In particular, CREB payed an important role, especially with the somatostatin promoter. The ability of these factors to de-repress partially assembled templates but not maximally assembled templates suggests that at least another step is required for depression. This step may include the activities of chromatin remodelling factors or spacing activities not present in the purified NAP-1 assembly system. Although Tax+CREB+CBP could de-repress the HTLV-I template, the overall level of activation was not as high as that demonstrated by other investigators (for example Kashanchi *et al*., 1998) and the activation seen with the S-150 extract or the N1/N2(H3,H4) extract (see section 5.2.2 and 5.2.3) in this work. This finding implies that interactions between Tax and other components of the cellular co-activator network , or basal transcription machinery, are required for high level Tax mediated LTR transactivation.

(d) Identification of a HTLV -I specific transcriptional activation activity.

The transcriptional activation activity identified in the *Xenopus* N1/N2(H3,H4) complex induced high level transcriptional activation of HTLV-I templates on both naked or chromatin assembled DNA. This finding may have significance with regards to HTLV-I transcription control for two reasons. Firstly, this finding demonstrates that HTLV-I transcription can be highly activated from nucleosome assembled templates. Secondly, the presence of such a factor may present an alternative mechanism for HTLV-I transcriptional activation. We provide preliminary evidence indicating that this activity is consistent with a DNA binding factor. The discovery of such an activation activity provides a platform for its identification and characterisation. Given the conservation of transcriptional activation mechanisms especially between vertebrates or metazoans, identification of the transcriptional activation activity may have important implications for the further characterisation of HTLV-I proviral expression.

(e) The identification of specific HMG-I(Y) binding sites on the HTLV-I LTR.

This study is the first to report the discovery of three potential HMG-I(Y) binding regions within a 147 bp fragment spanning regions -161 to -13 on the HTLV-I LTR. It is not clear at this stage whether these sites are important. However, the role of HMG-I(Y) in HTLV-I expression is suggested since preliminary investigations of the functional significance of this binding using anti-sense experiments, suggested that HMG-I(Y) plays a role in PMA inducible HTLV-I gene expression.

Implications and future work

Control of HTLV-I transcription in chromatin by CREB, Tax and CBP

This study has endeavoured to recreate an *in vitro* system for analysing HTLV-I transcription that resembles how the provirus is transcribed *in vivo*. Using this strategy, we hoped to make more accurate predictions of the transcriptional control of HTLV-I *in vivo*. To this end, we employed the natural promoters of cellular and viral genes rather than engineered templates designed to give high levels of transcription. We also used full length transcription factors rather than truncated or chimeric proteins. In terms of the future development of a purified chromatin assembly system, it will be important to add purified nucleosome spacing activity such as those identified by Gunchin *et al.* (2000) and

Tremethick and Frommer, (1992). Importantly, a system of chromatin assembly using purified components was developed so that the direct effect of Tax, CREB and CBP on inducible transcription could be determined. However, the very creation of such a purified system appears to be artificial in itself. As discussed in chapters 5 and 6, the NAP system may lack important components found in natural chromatin *in vivo*. However, this study has provided important information by showing that Tax, CREB and CBP are not sufficient for high level HTLV-I transcription on nucleosomal templates.

The ability of factors to induce transcription on sub-optimally assembled templates and not maximally assembled templates has interesting implications. The assembly of the templates by the NAP-1 system in the absence of natural spacing activities, could result in an artificially high nucleosome density and this possibility cannot be discounted. On the other hand, perhaps a more open chromatin structure is required for transcriptional activation of HTLV-I by Tax, CREB and CBP. Additional remodelling complexes may be required which may mimic sub-optimal levels of nucleosome density. Future work could include such remodellers.

As discussed in chapter 5, more analysis is required to elucidate the role of Tax, CREB and CBP on chromatin assembled templates. From the results presented in chapter 6, it appears that CREB pays a vital role in transcription, at least on chromatin templates assembled by the purified NAP-1 assembly system. Mayall *et al.* (1987) has shown that CREB is an important factor in TCRα transcription. Using the *Drosophila* S-190 assembly system, these investigators demonstrated that P-CREB in combination with Sp1 specifically disrupts the nucleosome array in the vicinity of the pTCRα enhancer. However, it is worth pointing out that this crude *Drosophila* extract is enriched in remodelling factors such as CRAC and NURF. Unlike the widely studied HIV-1 LTR, little is known about the detailed chromatin structure of the HTLV-I LTR. For example it is unknown whether inherent sequences position nucleosomes over the promoter, nor the effect of transacting factors on nucleosome positioning. Therefore, an important extension of this study could be to determine the nucleosome positioning over the promoter both in the transcriptionally repressed state and in response to activators. HTLV-I represents a unique model for such an investigation as phosphorylation of CREB is not required for activation. For example Mayall *et al.*, (1997) showed that P-CREB disrupted nucleosome structure. It would be interesting to determine whether the interaction between CREB and Tax is required for chromatin disruption as a step preceding transcriptional activation. Such an analysis could

be accomplished by micrococcal nuclease digestion of the assembled templates in the presence and absence of the factors followed by indirect end-labelling analysis. Determination of the nucleosome structure would correlate the important relationship between HTLV-I promoter structure and the ability of the promoter to function as a chromatin template.

Another useful approach to further elucidate the role of factors in transcriptional activation on chromatin templates would be to mutate the TRE elements to prevent the binding of CREB. Such mutations would confirm that CREB binding is necessary for transcriptional activation on chromatin templates. Similarly, the GC flanking regions of the HTLV-I promoters have been shown to be important for Tax recruitment *in vivo*. It would therefore be interesting to mutate these regions to determine if they are required for transcriptional activation on chromatin templates.

The role of CBP in gene expression

The study of CBP function has significant implications for the current theories concerning the role of CBP in gene expression. The identification of p300/CBP as a histone acetylase has led to the assumption that histone acetylation is a major mechanism by which CBP functions to activate transcription on chromatin templates *in vitro* and *in vivo*. In one popular and widely quoted theory, recruitment of CBP to an enhancer results in the enhancer specific acetylation of histones. Histone acetylation then destabilises the nucleosome structure, thereby resulting in transcription (Brown *et al.*, 2000). Such a mechanism of CBP activation is unlikely to have occurred in this study as we demonstrated that CBP activates transcription only from chromatin assembled templates both in the presence and absence of acetyl-coA. This observation provides evidence to suggest that CBP does not require its acetylation function to modestly activate transcription on sub-optimally assembled nucleosome templates. Many studies investigating the role of CBP in transcription have employed GAL4-CBP fusion proteins to target the HAT domain of CBP to artificial promoters. Many of these experiments are problematic, however, because several GAL-CBP fusion proteins appear to activate transcription more potently than the full length native protein (Swope *et al.*, 1996). Martínez-Balbás *et al.*, (1998) demonstrated that the CBP HAT domain tethered to a promoter by GAL4 activates transcription. Furthermore, these investigators showed by analysis of point mutations, that this transcriptional activation correlates with acetylation activity. However, the HAT domain of

CBP only stimulated transcription from certain promoters, namely the commonly used adenovirus E4 and major late promoters but not the adenovirus E1B promoter or the SV40 promoter. This study demonstrates that acetylation by CBP may not be required for the activation of all promoters. Moreover, several studies have demonstrated that acetyl-coA is not required for activation of gene transcription by CBP (Mayall *et al.*, 1997; Kashanchi *et al.*, 1998 Jenster *et al.*, 1998). Distinct from acetyltransferase activities, CBP has been shown to interact with the basal transcription factors TFIIB and TBP (Chrivia *et al.*, 1997; Parler *et al.,* 1996; Swope *et al.,* 1996). Such interaction with basal transcription factors may be the mechanism used by CBP to produce the modest activation of HTLV-I transcription in this study.

Our results do not imply that CBP/300 acetyltransferase activity is not important for transcription as there have been many reported studies that show that acetylation by CBP is necessary for function (Steger *et al.*, 1998; Martinez-Balbas *et al.*, 1998 Brown *et al.*, 2000). However, the mechanisms of acetylation mediated transcriptional activation may be more complex than the direct remodelling/destabilisation of chromatin structure. Here, we demonstrate that the activation of HTLV-I in response to CBP is not as high as anticipated. Indeed, the high level transcription from HTLV-I in the presence of the N1/N2 (H3,H4) complex (section 5.2.4) and S-150 extract (section 5.2.2) suggests that higher levels of transcription activation can occur. This observation suggests that CBP recruitment is not sufficient for high-level transcriptional activation on chromatin templates. Importantly, the experiments conducted in this study employed a purified nucleosome assembly system free from co-activators and modulators of chromatin structure. Therefore, the acetylation activities of CBP may be required for the expected high level activation of HTLV-I templates, however, only in the presence of other factors. With regard to the HTLV-I promoter, the acetylation activity of CBP (in combination with appropriate factors) and interactions with the basal transcription machinery may both be involved in transcriptional control. Indeed, Jenser *et al.* (1999) have shown a dual role for co-activators in steroid receptor induction of gene transcription, chromatin remodelling and enhanced stabilisation of the pre-initiation complex.

With regard to the mechanism of CBP in cellular gene expression, CBP is a very large protein with many binding and docking domains for a diverse array of transcription factors (see figure 1.7). It is a rather simplistic view that these factors all function solely to recruit CBP to the promoter which then activates transcription of all genes in a similar fashion. There must be mechanisms present in the cell to regulate the precise levels of transcription

both in a positive and negative manner. Because of the tight control of transcriptional regulation, it is unlikely that CBP alone is the sole co-factor for all genes. It is reasonable to suggest that many other proteins and protein complexes such as chromatin remodelling machines, architectural proteins and acetylation machines are recruited to specific promoters to enable the precise regulation of genes. In this way, CBP provides a mechanism for the integration of diverse signalling pathways. Therefore, based on the evidence that on pure nucleosome templates, histone acetylation is not sufficient for CBP mediated activation, we suggest that the acetylase activity of CBP may be involved in transcription but through mechanisms distinct from histone acetylation.

CBP has been identified as a component of the mammalian RNA polymerase II holoenzyme. Interestingly another component of the holoenzyme is the BRG1 subunit of the chromatin remodelling complex SWI/SNF (Neish *et al.*, 1998). Therefore, CBP may serve to recruit the holoenzyme and /or ATP dependent chromatin remodelling machines. CBP has also been shown to recruit other HAT enzymes p/CAF and SRC1 to form a multi-HAT/activator enhancer complex (Yao *et al.*, 1996; Chen *et al.*, 1997; Spencer *et al.*, 1997). This association suggests that a combination of acetyltransferase activities may be required for certain functions. One of the mechanisms by which these enzymes could function differently is by having distinct histone targets. For example, p/CAF acetylates H3 and H4 *in vitro* (Yang *et al.*, 1996) whereas CBP acetylates all four core histones equally well (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996 and chapter 3 of this work). This apparent redundancy might reflect synergistic or differential acetylation roles on certain promoters (section 1.4.2.3; Schiltz *et al.*, 1999; Kurokawa *et al.*, 1998). The acetylation of non-histone targets such as transcription factors and co-factors, demonstrated in chapter 3 of this work and by many other studies, provides perhaps the best evidence to suggest that other factors are involved in CBP function. Indeed recruitment of CBP to the promoter of some genes has been shown by the ChIP assay to not result in transcriptional activation by histone acetylation, rather, acetylation of transcription factors by CBP contributes to the activation (Dr Thanos, personal communication). Furthermore, acetyl-coA has been recently shown to increase *in vitro* transcription in the absence of histone proteins (Galasinski *et al.*, 2000). Finally, a separate mechanism exists whereby the function of CBP is differentially regulated. For example, Perissi *et al.* (1999) has demonstrated that E1A binding to the CH3 domain of CBP (figure 1.7) inhibits acetyltransferase activity whereas the co-factor p/CIP can either inhibit or stimulate CBP acetyltransferase activity depending on the substrate. Furthermore, Herrera *et al.* (1997) also demonstrated that the HAT activity of GCN5 and p/CAF can be stabilised by co-

enzymes. These interactions may result in different effects of CBP function for different classes of transcriptional regulators and different promoters. This regulatory role of factors in the function of CBP activation could be examined using the NAP-1 assembly system.

A further exciting application of the HTLV-I model system is to elucidate the function of PKA in transcriptional control. It was once believed that CREB phosphorylation was sufficient for activation by enabling the recruitment of CBP. However, there has been recent controversy in the literature regarding the role of PKA phosphorylation in CREB mediated gene activation. For example, in PC12 cells and primary neurons, depolarisation activates the *c-fos* CRE in a manner that is blocked by inhibitors of PKA even though CREB remains phosphorylated (Impey *et al.*, 1994; Thompson *et al.*, 1995). However, the mechanism by which PKA contributes to activation is unknown. PKA may phosphorylate components of the general transcriptional machinery or transcription factors downstream from CREB. Xu *et al.*, (1998) have suggested that PKA phosphorylated CBP directly while Zanger *et al.*, (1999) have argued that PKA might effect a step downstream from CBP. On the other hand, a recent study by Cardinaux *et al.* (2000) has shown that recruitment of CBP is sufficient for CREB mediated gene activation by creating CREB mutants that constitutively bind CBP in the absence of PKA. The HTLV-I model appears to represent a unique situation whereby CREB phosphorylation can be dissociated from CBP recruitment as CREB does not need to be phosphorylated in order to recruit CBP in the presence of Tax (Kwok *et al.*, 1996). In this study we show that CREB, in the presence of Tax and CBP without PKA results in the modest activation of HTLV-I transcription. It would be interesting to incorporate PKA into the reaction to determine if PKA is involved in the activation of HTLV-I on chromatin assembled templates.

A model for the direct role of chromatin in inducible transcription

Until recently, nucleosome assembly was thought of as a general represser of basal transcription. However, the assembly of promoters into nucleosomes imposes an addition layer of regulation on the transcriptional control of some genes. This regulatory role may involve physically bringing enhancer and promoter elements into contact with the basal transcription machinery or promoting interactions among transcription factors by acting as a scaffold. More recently it has been suggested by some investigators that nucleosomes themselves may be involved directly in the regulation process. One hypothesis is that the nucleosomes act as targets to direct regulatory complexes to specific promoters. In such a

model for transcriptional activation, co-factors play a central role (Strahl and Allis, 2000 Winston and Allis, 1999).

In light of the current evidence with regard to transcriptional control and the results of this research study, a model can be proposed to explain the ability of the cell to precisely control both activation and down regulation of transcription. This model is shown diagrammatically in figure 7.1. Previously, many models attempting to explain the complex process of transcriptional regulation viewed DNA as equivalent to a piece of string decorated with transcription factors located strategically at regulatory promoter regions with mysterious loops linking distantly located factors. However, the DNA template for transcription *in vivo* exists in highly organised nucleosome structures. Therefore in our proposed model, the first step in transcriptional activation is the binding of sequence specific transcription factors to their recognition sites on the nucleosome in response to signalling cascades. In the case of HTLV-I transcription, this equates to CREB binding to the nucleosome as demonstrated in chapter 4. On some genes, this step may involve the binding of many transcription factors that create a combined interaction interface.

The sequence specific transcription factors then recruit the first round of co-factors directly to the promoter. First round cofactors are recruited by protein-protein interactions between the activation domains of sequence specific transcription factors and co-factors that comprise components of chromatin remodellers such as CBP or SAGA. The recruitment of these co-factors may directly bring about transcriptional activation or repression through modulation of chromatin structure by post-translational acetylation or phosphorylation. However, a primary modifying role for some of these co-factors may be to directly label the nucleosome. Indeed Strahl and Allis, (2000) have suggested that a histone code operates in the cell. This code is comprised of combinations of post-translational modifications including acetylation, phosphorylation, ubiquitination, methylation or ADP-ribosylation (see figure 7.2). Using HTLV-I as an example, Tax and CBP constitute the first round of co-factor recruitment.

The nucleosomes marked with the histone code then act as direct targets to recruit a second round of co-factors that read the histone code. Whereas the first wave co-factors are recruited to the promoter by protein-protein interactions with transcription factors, second wave co-factors are recruited directly to the promoter by recognition of the histone code. This second wave may consist of ATP dependent re-modelling activities or other nucleosome modification complexes. The second wave of co-factors may function to

Figure 7.1: The sequential cofactor recruitment hypothesis. Step 1: Inducible transcription is initiated by binding or activation (for example, by phosphorylation) of a sequence specific transcription factor or factors to their recognition sites in chromatin. **Step 2:** Co-factors are recruited directly to the transcription factor by protein-protein interactions between the activation domain of the activator and co-factors. Recruitment of co-factors may lead directly to transcriptional activation or silencing by chromatin remodelling (at **step 5**). **Step 3:** Alternatively, the recruited co-factors may label the histone tails of the core histones with the 'histone code' (figure 7.1). **Step 4:** successive waves of co-factors may be recruited directly to the labelled histones (figure 7.1). Subsequent waves of co-factor recruitment may result in specific downstream events such as transcriptional activation or silencing (**step 5**) and may also label the histone tails with a different histone code to recruit further co-factors with specialised functions. There may be many successive rounds of co-factor recruitment in order to control the precise levels of transcription.
Importantly, modifications of the co-factors themselves may also modulate protein-protein interactions and provideanother potential mechanism of regulation (see text).

1

Binding or activation of
sequence specific
Transcription factor(s)
(via cell signalling pathways)

2

First wave of co-factor recruitment
(specific protein-protein
contacts between
Transcription factor(s) and
co-factors)

3

Labelling of histone tails
with the 'histone code'

Ac
P
Ac

4

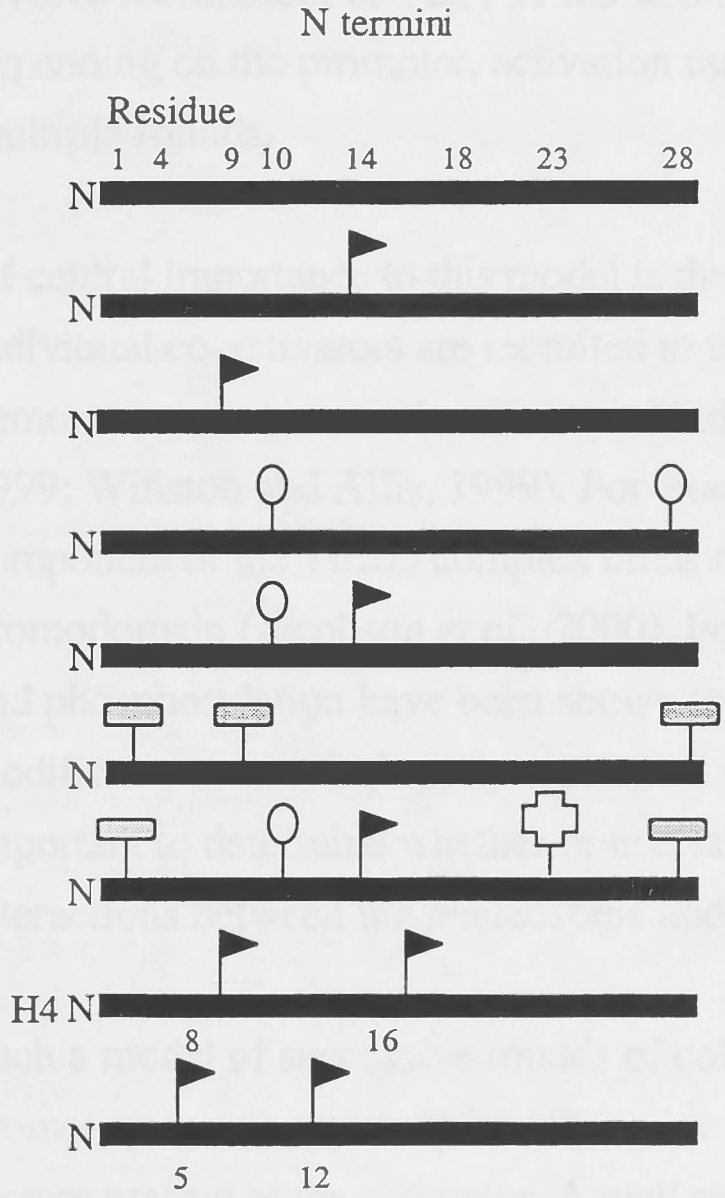
Successive rounds of
co-factor recruitment

Me
P
Ac

Histone tails labelled
with a different code

5

Specific downstream
Effects
(eg transcription)



Modification state	Associated Protein/molecule	Function
Unmodified	Sir3/Sir4/Tup1	Silencing
Acetylated	Bromodomain	Transcription
Acetylated	?	Histone deposition?
Phosphorylated	SMC/ Condensins	Mitosis/meiosis
Phos/acetyl	?	Transcription
Methylated	?	Transcription?
Higher-order combinations	?	?
Acetylated	?	Transcription
Acetylated	RCAF?	Histone deposition

Figure 7.2: The histone code hypothesis. Histone modifications such as acetylation (⚑), phosphorylation (♀) or methylation (⊤) occur at selected residues on core histones (depicted here are H3, H4). Some combinations have been closely linked to a biological event such as transcription. The histone code hypothesis suggests that multiple histone modifications, acting in combinatorial or sequential fashion on one or multiple histone tails specify unique downstream functions. Relevant proteins or protein domains that are known to interact or associate with distinct modifications are indicated (Strahl and Allis, 2000 and source of the figure).

activate transcription by chromatin remodelling or again mark the histone tails for a third round of coactivator recruitment. As illustrated in figure 7.2, complex combinations of modifications are possible. In some cases, remodelling complexes may interact first to allow post-translational modification. Using the HTLV-I example, the second round of cofactors may not have been present in the purified NAP-1 assembly system and therefore, the activation process stops at the first recruitment step and does not enable high level transcription. Successive recruitment of cofactors may continue until a specific functional state is reached. For example in the case of transcriptional activation, the final steps may involve recruitment of TBP, TFIID and the RNA pol II holoenzyme. Importantly, depending on the promoter, activation may require one round of co-factor recruitment or multiple rounds.

Of central importance to this model is the identification of the mechanisms in which individual co-activators are recruited to the code on the histone tails. Recent reports demonstrate that bromodomains can bind specifically to acetylated histones (Dhalluin *et al.*, 1999; Winston and Allis, 1999). For example, it has recently been shown that the $TAF_{II}250$ component of the TFIID complex binds to multiply acetylated histones through its bromodomain (Jacobson *et al.*, 2000). Furthermore, the dual modifications of acetylation and phosphorylation have been shown to occur on the same histone H3 tail and that this modification event is directly associated with active genes (Clayton *et al.*, 2000). It will be important to determine whether or not labelling of the histone H3 tail in this way regulates interactions between the nucleosome and co-factors.

Such a model of successive rounds of cofactor recruitment may explain the switching of promoters from a transcriptionally active template to a silent state by exchanging the co-factors present at the promoter. A new set of co-factors may label histones with different combinations of post-translational modifications thereby recruiting co-factors to set up silencing pathways. For example the yeast silencing protein Tup1/Ssn6 binds to unacetylated or monoacetylated histones tails more strongly when compared with its binding to hyperacetylated H3 or H4 (Edmondson *et al*, 1996). An important question is what triggers the switch in co-factor compliment at the enhancer? One way this could occur is through protein-protein interactions between co-factors mediated by modifications such as acetylation. Many transcription factors have been identified as substrates of acetylation by CBP. These factors include CREB, AP-1, histone H1, PC4 and HMG proteins in this study (see section 3.2.4.2) and many other factors (see Brown *et al.*, 2000). Just as specific co-factors are recruited to acetylated histones, they may also be recruited to

acetylated transcription factors. The acetylation of transcription factors may recruit a different set of cofactors by protein-protein interactions that are specific for transcriptional repression. These factors may themselves down regulate transcription by chromatin remodelling. Alternatively, these newly recruited factors may also re-label the histone tails, changing the histone code such that cofactors involved in transcriptional repression are recruited to the promoter such as Tup1/Ssn6. A final stage in silencing may be reached when the histones encode for the recruitment of DNA methylases which set up regions of silenced chromatin. In addition to the post-translational modification of transcription factors recruiting co-factors, acetylation or other forms of post-translational modification may regulate the formation of the enhancer complex. For example, acetylation of HMG-17 by p/CAF and acetylation of HMG-14 by p300 alters the interactions of these HMG proteins with nucleosomes (Herrera *et al.*, 1999; Bergel *et al.*, 2000). Similarly, acetylation or phosphorylation of HMG-I(Y) influences its ability to interact with nucleosome core particles (Banks *et al.*, 2000; Yie *et al.*, 1999; Munshi *et al.*, 1998). In the case of the INFβ enhancersome, acetylation of HMG-I(Y) results in dissociation of HMG-I(Y) from the enhancer and a down regulation of transcription (Munshi *et al.*, 1998).

The scenario just described results in transcriptional repression. However, post-translational modification of transcription factors and co-factors may also recruit factors involved with other specific down-stream functions such as transcription. Using the HTLV-I example, in chapter 3 we showed that CBP acetylated CREB but this acetylation had no effect on DNA binding. It may be possible that acetylation of CREB by CBP (or a factor other than CBP) influences its interaction with co-factors. In this way, a specific co-factor compliment could be formed at the HTLV-I promoter by protein-protein interactions. This model of successive targeted co-factor recruitment in different combinations may explain how a relatively small number of transcriptional regulatory proteins function to achieve the enormous diversity in gene expression required for the development of eukaryotic organisms.

Clearly, this model requires further investigation to determine its applicability to *in vivo* transcriptional control. The NAP-1 chromatin assembly and transcription system developed here provides a unique opportunity to dissect the mechanisms involved in transcription because chromatin assembly can be separated from co-factor activity. ChIP assays could be used to determine the order of factor recruitment and their acetylation states in a an

analogous way to that on the yeast homothallic promoter (Cosma *et al.*, 1999; Krebs *et al.*, 1999).

Finally, the work in this thesis has important implications for the control of HTLV-I viral transcription. Since HTLV-I exists within the context of chromatin, the virus must have evolved and conserved mechanisms which enable gene activation within a normally repressive environment. Here, we showed that a combination of the virally expressed protein Tax, and the cellular expressed factors CBP and CREB, are only partially sufficient for high level transcriptional activation within the context of chromatin. We propose that co-factors missing from the purified system, are involved in high level expression of the virus. The viral co-factor, Tax may be involved (by protein-protein interactions) in the recruitment of an HTLV-I LTR specific compliment of co-factors that ultimately lead HTLV-I down the pathway to high level transcriptional activation. An interesting, but poorly understood element of HTLV-I mediated disease is the mechanisms which control viral latency. Perhaps the trigger to end the latency period is a unique co-factor or combination of cofactors. Indeed, the identification of a transcriptional activation activity specific for HTLV-I in *Xenopus* oocytes (section 5.2.3) suggests the presence of such a transcriptional activator. Use of the NAP-1 assembly system as a tool to identify and define factors that cause transcriptional activation may help in the understanding of viral infection and disease progression.

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